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**United States Patent** [19]

Daynes et al.

[11] **Patent Number:** 5,562,910[45] **Date of Patent:** \* Oct. 8, 1996[54] **VACCINE COMPOSITIONS AND METHOD FOR ENHANCING AN IMMUNE RESPONSE**[75] **Inventors:** Raymond A. Daynes, Park City;  
Barbara A. Araneo, Salt Lake City,  
both of Utah[73] **Assignee:** University of Utah Research  
Foundation, Salt Lake City, Utah[\*] **Notice:** The portion of the term of this patent  
subsequent to Sep. 9, 2013, has been  
disclaimed.[21] **Appl. No.:** 123,843[22] **Filed:** Sep. 9, 1993**Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 13,972, Feb. 4, 1993, abandoned, and a continuation-in-part of Ser. No. 779,499, Oct. 18, 1991, abandoned, which is a continuation-in-part of Ser. No. 412,270, Sep. 25, 1989, abandoned.

[51] **Int. Cl.<sup>6</sup>** ..... A61K 39/00; A61K 31/56;  
A61K 31/59[52] **U.S. Cl.** ..... 424/278.1; 424/209.1;  
424/211.1; 424/217.1; 424/219.1; 424/224.1;  
424/225.1; 424/230.1; 424/231.1; 424/244.1;  
424/245.1; 424/247.1; 424/254.1; 424/256.1;  
514/169; 514/725; 514/885; 514/171; 514/178;  
514/167[58] **Field of Search** ..... 424/88, 85.1, 278.1,  
424/225.1, 209.1, 245.1, 247.1, 254.1, 212.1,  
219.1, 217.1, 244.1, 231.1, 211.1, 256.1,  
230.1, 224.1, 184.1, 256.1, 230.1, 224.1,  
184.1; 514/885, 167, 169, 178, 725, 885,  
171[56] **References Cited****U.S. PATENT DOCUMENTS**

4,963,354	10/1990	Shepard et al.	424/85.1
5,077,284	12/1991	Loria et al.	514/171
5,098,899	3/1992	Gilbert et al.	514/167
5,206,008	4/1993	Loria	424/45
5,407,684	4/1995	Loria et al.	424/442

**FOREIGN PATENT DOCUMENTS**

2204237	11/1988	United Kingdom
WO91/04030	4/1991	WIPO

**OTHER PUBLICATIONS**Bienenstock, J. et al. (1983). "Regulation of Lymphoblast Traffic and Localization in Mucosal Tissues, with Emphasis on IgA," *Federation Proceedings* 42:3213-3217.Kawanishi, H. et al. (1983). "Mechanisms Regulating IgA Class-Specific Immunoglobulin Production in Murine Gut-Associated Lymphoid Tissue," *J. Exp. Med.* 157:433-450.Kiyono, H. et al. (1984). "Isotype Specificity of Helper T Cell Clones," *J. Exp. Med.* 159:798-811.Weindruch, R. et al. (1984). "Food Intake Reduction and Immunologic Alterations in Mice Fed Dehydroepiandrosterone," *Exp. Gerontol.* 19:297-304 (1984).Komori, T. et al. (1985). "The Effect of 1 $\alpha$ -Hydroxyvitamin D<sub>3</sub> on Primary Antibody Formation in Mice," *Immunopharmacol.* 9:141-146.Tabata, T. et al. (1986). "The Effect of 1 $\alpha$ -Hydroxyvitamin D<sub>3</sub> on Cell-Mediated Immunity in Hemodialyzed Patients," *J. Clin. Endocrin. Metab.* 63:1218-1221.Dinarello, C. A. et al. (1987). "Current Concepts: Lymphokines," *New Eng. J. Med.* 317:940-945.Mestecky, J. (1987). "The Common Mucosal System and Current Strategies for Induction of Immune Responses in External Secretions," *J. Clin. Immunol.* 7:265-276.Nossal, G. J. V. (1987). "Current Concepts: Immunology: The Basic Components of the Immune System," *New Eng. J. Med.* 316:1320-1325.Petkovich, P. M. et al. (1987). "1,25-Dihydroxyvitamin D<sub>3</sub> Increases Epidermal Growth Factor Receptors and Transforming Growth Factor  $\beta$ -Like Activity in a Bone-Derived Cell Line," *J. Biol. Chem.* 262:13424-13428.Pfeilschifter, J. et al. (1987). "Modulation of Type  $\beta$  Transforming Growth Factor Activity in Bone Cultures by Osteotropic Hormones," *Proc. Natl. Acad. Sci. USA* 84:2024-2048.Rigby, W. F. C. (1988). "The Immunobiology of Vitamin D," *Immunol. Today* 9: 54-57.

(List continued on next page.)

**Primary Examiner**—James C. Housel**Assistant Examiner**—Julie Krsek-Staples**Attorney, Agent, or Firm**—Venable, Baetjer, Howard & Civiletti, LLP[57] **ABSTRACT**

The invention relates to a vaccine which comprises an antigen and an immune response augmenting agent. The immune response augmenting agent is capable of enhancing T cell lymphokine production. Suitable immune response augmenting agents include, but are not limited to, dehydroepiandrosterone (DHEA) and DHEA-derivatives. Examples of DHEA derivatives include DHEA-sulfate (DHEA-S), 16 $\alpha$ -bromo-DHEA, 7-oxo-DHEA, 16 $\alpha$ -bromo-DHEA-S and 7-oxo-DHEA-S.

The invention also relates to a method for enhancing a vaccine-induced humoral immune response which comprises administering a vaccine which comprises an antigen and an immunomodulator. The immunomodulator may be an immune response augmenting agent, a lymphoid organ modifying agent or a mixture of the immune response augmenting agent and lymphoid organ modifying agent. Suitable lymphoid organ modifying agents include, but are not limited to, 1,25-dihydroxy Vitamin D<sub>3</sub>, biologically active Vitamin D<sub>3</sub> derivatives which are capable of activating the intracellular Vitamin D<sub>3</sub> receptor, all trans-retinoic acid, retinoic acid derivatives, retinol, retinol derivatives and glucocorticoid. Alternatively, the method for enhancing a vaccine-induced humoral immune response comprises separately administering the immunomodulator and a vaccine containing an antigen.

**36 Claims, 18 Drawing Sheets**

## OTHER PUBLICATIONS

- Weinberg, A. et al. (1988). "Recombinant Interleukin 2 as an Adjuvant for Vaccine-Induced Protection," *J. Immunol.* 140:294-299.
- Abc, J. et al. (1989). "A Synthetic Analogue of Vitamin D<sub>3</sub> 22-Oxa-1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub>, is a Potent Modulator of In vivo Immunoregulating Activity Without Inducing Hypercalcemia in Mice," *Endocrinol.* 124: 2645-21647.
- Bhalla, A. K. (1989). "Hormones and the Immune Response," *Ann. of Rheumatic Dis.* 48:1-6.
- Coffman, R. L. (1989). "T Helper Heterogeneity and Immune Response Patterns," *Hosp. Practice* (Aug. 15, 1989):101-133.
- Coffman, R. L. et al. (1989). "Transforming Growth Factor  $\beta$  Specifically Enhances IgA Production by Lippolysaccharide-Stimulated Murine B Lymphocytes," *J. Exp. Med.* 170:1039-1044.
- Daynes, R. A. et al. (1989). "Constrasting Effects of Glucocorticoids on the Capacity of T Cells to Produce the Growth Factors Interleukin 2 and Interleukin 4," *Eur. J. Immunol.* 19:2319-2325.
- Hahn-Zoric, M. et al. (1989). "The Influence on the Secretory IgA Antibody Levels in Lactating Women of Oral Typhoid and Parental Cholera Vaccines Given Alone or in Combination," *Scand. J. Infect. Dis.* 21:421-426.
- Nunberg, J. H. et al. (1989). "Interleukin 2 Acts as an Adjuvant to Increase the Potency of Inactivated Rabies Virus Vaccine," *Proc. Natl. Acad. Sci. USA* 86 4240-4243.
- Daynes, R. A. et al. (1990). "Regulation of Murine Lymphokine Production In Vivo: II. Dehydroepiandrosterone is a Natural Enhancer of Interleukin 2 Synthesis by Helper T Cells," *Eur. J. Immunol.* 20:793-802.
- Daynes, R. A. et al. (1990). "Regulation of Murine Lymphokine Production In Vivo: III. The Lymphoid Tissue Microenvironment Exerts Regulatory Influences Over T Helper Cell Function," *J. Exp. Med.* 171:979-996.
- Finkelman, F. D. et al. (1990). "Lymphokine Control of In Vivo Immunoglobulin Isotype Selection," *Ann. Rev. Immunol.* 8:303-333.
- Lebman, D. A. et al. (1990). "Molecular Characterization of Germ-Line Immuno-globulin A Transcripts Produced During Transforming Growth Factor Type  $\beta$ -Induced Isotype Switching," *Proc. Natl. Acad. Sci. USA* 87:3962-3966.
- Mbawuke, I. N. et al. (1990). "Enhancement of the Protective Efficacy of Inactivated Influenza A Virus Vaccine in Aged Mice by IL-2 Liposomes," *Vaccine* 8 347-352.
- Risdon, G. et al. (1990). "Mechanisms of Chemoprevention by Dietal Dehydroisro androsterone: Inhibition of Lymphopoiesis," *Am.J. Pathol.* 136:759-769.
- Araneo, B. A. et al. (1991). "Dihydrotestosterone Exerts a Depressive Influence on the Production of Interleukin-4 (IL-4), IL-5, and  $\gamma$ -Interferon, But Not IL-2 by Activated Murine T Cells," *Blood* 78:688-699.
- Meikle, A. W. et al. (1991). "Adrenal Androgen Secretion and Biologic Effects," *Endocrin. Metab. Clin. N. Am.* 20:381-400.
- Quiding, M. et al. (1991). "Intestinal Immune Responses in Humans," *J. Clin. Invest.* 88:143-148.
- Suzuki, T. et al. (1991). "Dehydroepiandrosterone Enhances IL2 Production and Cytotoxic Effector Function of Human T Cells," *Clin. Immunol. Immunopathol.* 61:202-211.
- Wiedmeier, S. E. et al. (1991). "Thymic Modulation of IL-2 and IL-4 Synthesis by Peripheral T Cells," *Cell. Immunol.* 135:501-518.
- Wu, C. Y. et al. (1991). "Glucocorticoids Suppress the Production of Interleukin 4 by Human Lymphocytes," *Eur. J. Immunol.* 21:2645-2647.
- Abraham, E. et al. (1992). "Intranasal Immunization with Liposomes Containing IL-2 Enhances Bacterial Polysaccharide Antigen-Specific Pulmonary Secretory Antibody Response," *J. Immunol.* 149:3719-3726.
- Binderup, L. et al. (1992). "Commentary: Immunological Properties of Vitamin D Analogues and Metabolites," *Biochem. Pharmacol.* 43:1885-1892.
- Daynes, R. A. et al. (1992). "Natural Regulators of T-Cell Lymphokine Production In Vivo," *J. Immunother* 12:174-179.
- Defrance, T. et al. (1992). "Interleukin 10 and Transforming Growth Factor  $\beta$  Co-Operate to Induce Anti-CD40-Activated Naive [sic.] Human B Cells to Secrete Immunoglobulin A," *J. Exp. Med.* 175:671-682.
- Hewison, M. (1992). "Vitamin D and the Immune System," *J. Endocrinol.* 132: 173-175.
- Lemire, J. M. (1992). "Immunomodulatory Role of 1,25-Dihydroxyvitamin D<sub>3</sub>," *J. Cell. Biochem.* 49:26-31.
- Müller, K. et al. (1992). "Inhibition of Human T Lymphocyte Proliferation and Cytokine Production by 1,25-Dihydroxyvitamin D<sub>3</sub>, Differential Effects on CD45RA+ and CD45RO+ Cells," *Autoimmunity* 14:37-43.
- Rasmussen, K. R. et al. (1992). "Dehydroepiandrosterone-Induced Reduction of *Cryptosporidium parvum* Infections in Aged Syrian Golden Hamsters," *J. Parasitol.* 78:554-557.
- Roberts, A. B. et al. (1992). "Mechanistic Interrelationships Between Two Super-families: The Steroid/Retinoid Receptors and Transforming Growth Factor- $\beta$ " *Cancer Surveys* 14:205-220.
- Shull, M. M. et al. (1992). "Targeted Disruption of the Mouse Transforming Growth Factor- $\beta$ -1 Gene Results in Multifocal Inflammatory Disease," *Nature* 359:693-699.
- van Vlasselaer, P. et al. (1992). "Transforming Growth Factor- $\beta$  Directs IgA Switching in Human B Cells," *J. Immunol.* 148:2062-2067.
- Araneo, B. A. et al. (1993). "Administration of Dehydroepiandrosterone to Burned Mice Preserves Normal Immunologic Competence," *Arch. Surg.* 128:318-325.
- Araneo, B. A. et al. (1993). "Reversal of the Immunosenescent Phenotype by Dehydroepiandrosterone: Hormone Treatment Provides an Adjuvant Effect on the Immunization of Aged Mice with Recombinant Hepatitis B Surface Antigen," *J. Infect Dis.* 167:830-840.
- Casson, P. R. et al. (1993). "Oral Dehydroepiandrosterone in Physiologic Doses Modulates Immune Function in Postmenopausal Women," *Am. J. Obstet. Gynecol.* 169:1536-1539.
- Garg, M. et al. (1993). "Reversal of Age-Associated Decline in Immune Response to Pnu-Immune Vaccine by Supplementation with Steriod Hormone Dehydroepiandrosterone," *Infect. Immun.* 61:2238-2241.
- Daynes, R. A. and Araneo, B. A. (1992). "Prevention and Reversal of some Age-Associated Changes in Immunologic Responses by Supplemental Dehydroepiandrosterone Sulphate Therapy," *Aging: Immunology and Infectious Disease* 3:135-154.
- Weksler, M. E. (1993). "Immune senescence and adrenal steroids: immune dysregulation and the action of dehydroepiandrosterone (DHEA) in old animals," *Eur. J. Clin. Pharmacol.* 45[Suppl 1]:S21-S23.

- Daynes, R. A. et al. (1991). "Locally active steroid hormones may facilitate compartmentalization of immunity by regulating the types of lymphokines produced by helper T cells," *Research in Immunology* 142:40-45.
- Glick et al, *Cell Regulation*; 1(1):87-97, 1989.
- McBride et al, *Vaccine* 6:414-418, 1988.
- Liew et al, *Eur. J. Immunol* 14:350-356, 1984.
- Finkelman et al, *Proc Natl Acad Sci*; 88:3657-3660, 1991.
- Aynaslar Batuman et al *J. Clin. Invest* 88:1574-1580, 1991.
- Holmgren J., *FEMS Microbiol Immunol* 84:1-10, 1991.
- Ernst et al, "Immunity in Mucosal Tissues" In *Basic & Clinical Immunology* 6th ed., Stitctta et al eds, Appleton & Lang, Norwalk, CT 1987 pp. 159-166.
- Van der Wall Bake, et al *Cellular Immunology* 144:417-428, 1992.
- Lebman et al, *J. Immunol.* 144:952-959, 1990.
- Binderrup L., *Biochemical Pharmacology* 43(9):1885-1892, 1992.
- Bikle DD, *Endocrine Reviews* 13(4):765-784, 1992.
- Loria et al, *Ann. N.Y. Acad. Sci* 650:363-366, 1992.
- Dawson et al, *J. Med Chem* 24:583-592, 1981.
- Dawson et al, *J. Med Chem* 24:1214-1223, 1981.
- Granner D. K. "Hormones of the Adrenal Cortex" *Harper's Biochemistry* Murray et al eds, Appleton & Lang Norwalk CT, 1988 pp. 511-523.
- Harper's Biochemistry*, Murray et al Eds, Appleton & Lange Norwalk CT, 1988 pp. 507-508.
- The Merck Index*, Merck & Co. Inc, Rahway, N.J. 9th Ed, 1976, pp. 1289-1291.

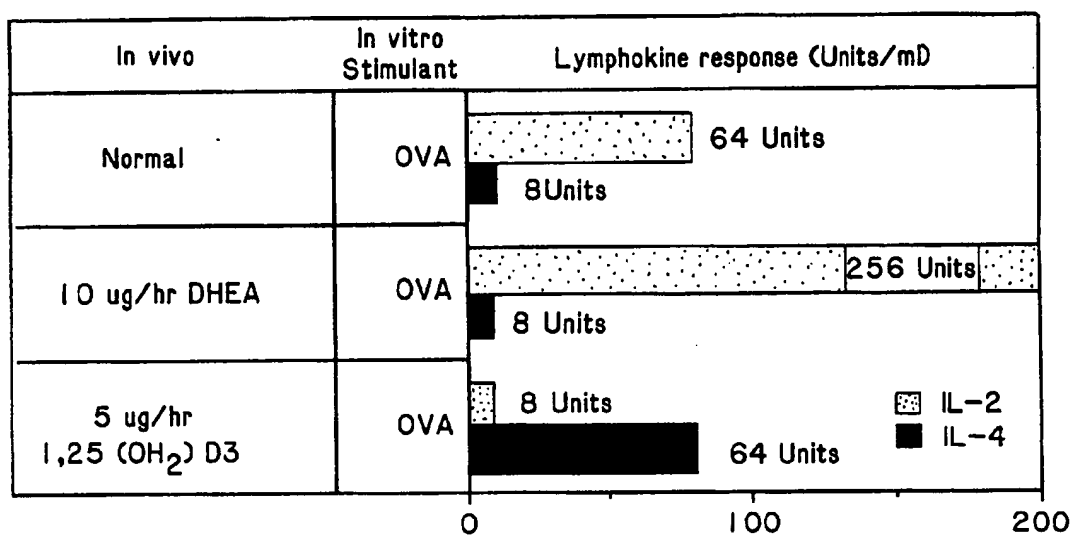
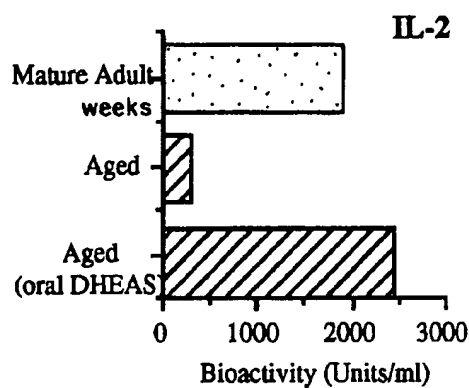
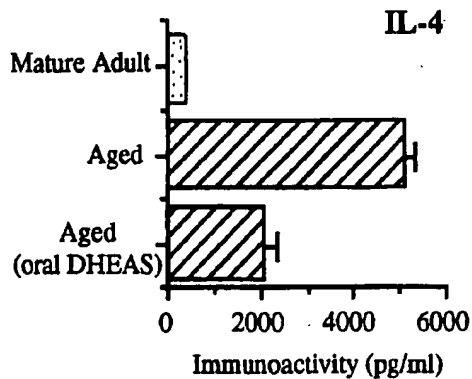
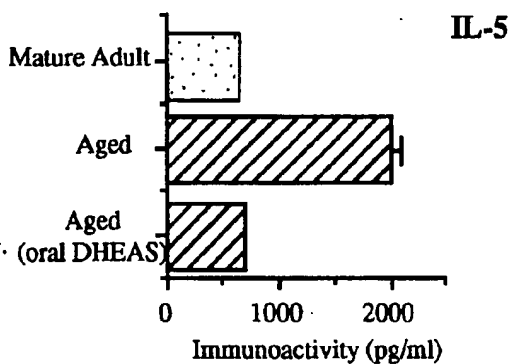
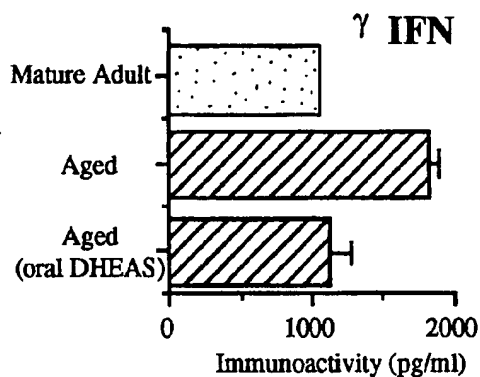
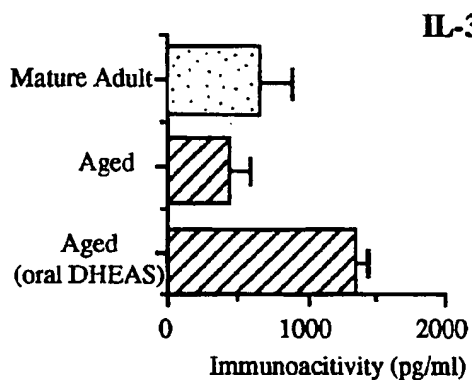
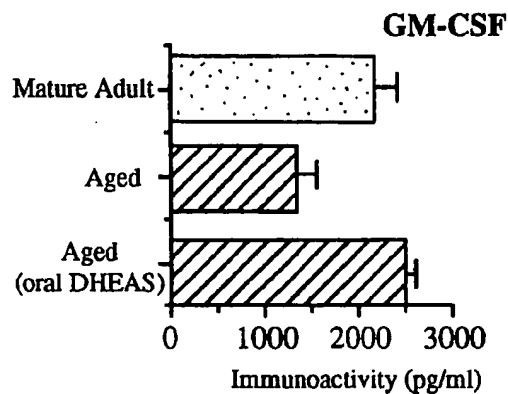


FIG.1

**FIG.2A****FIG.2B****FIG.2C****FIG.2D****FIG.2E****FIG.2F**

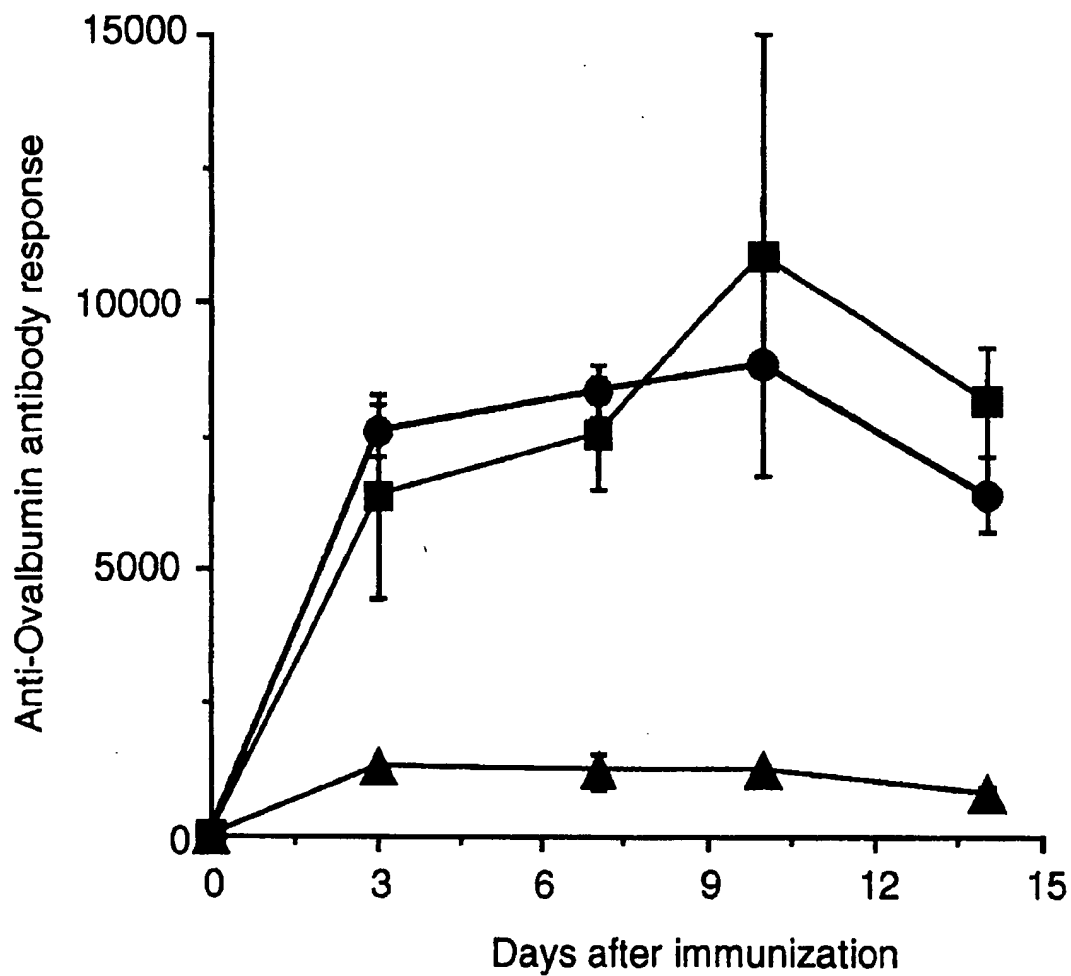
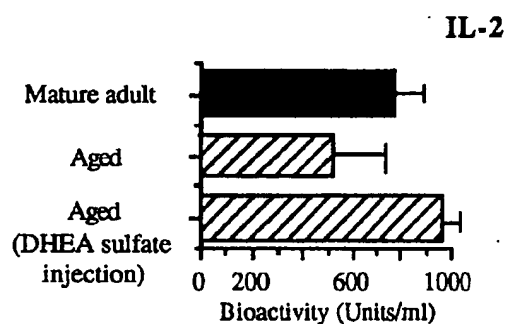
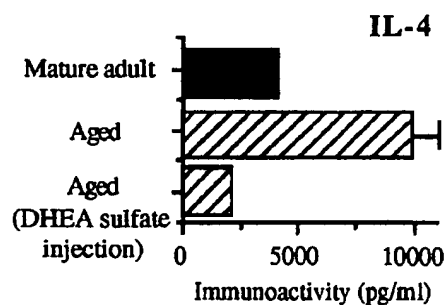
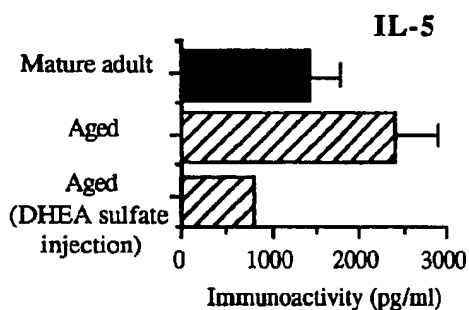
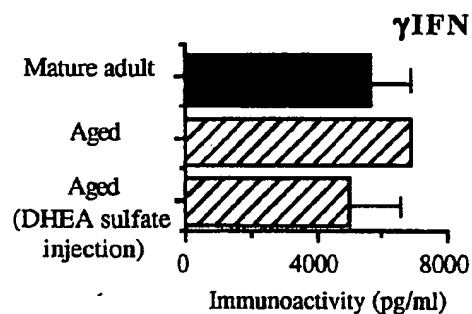
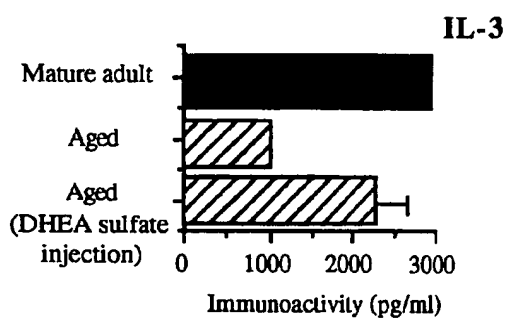
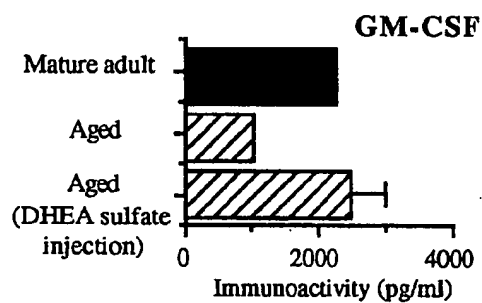
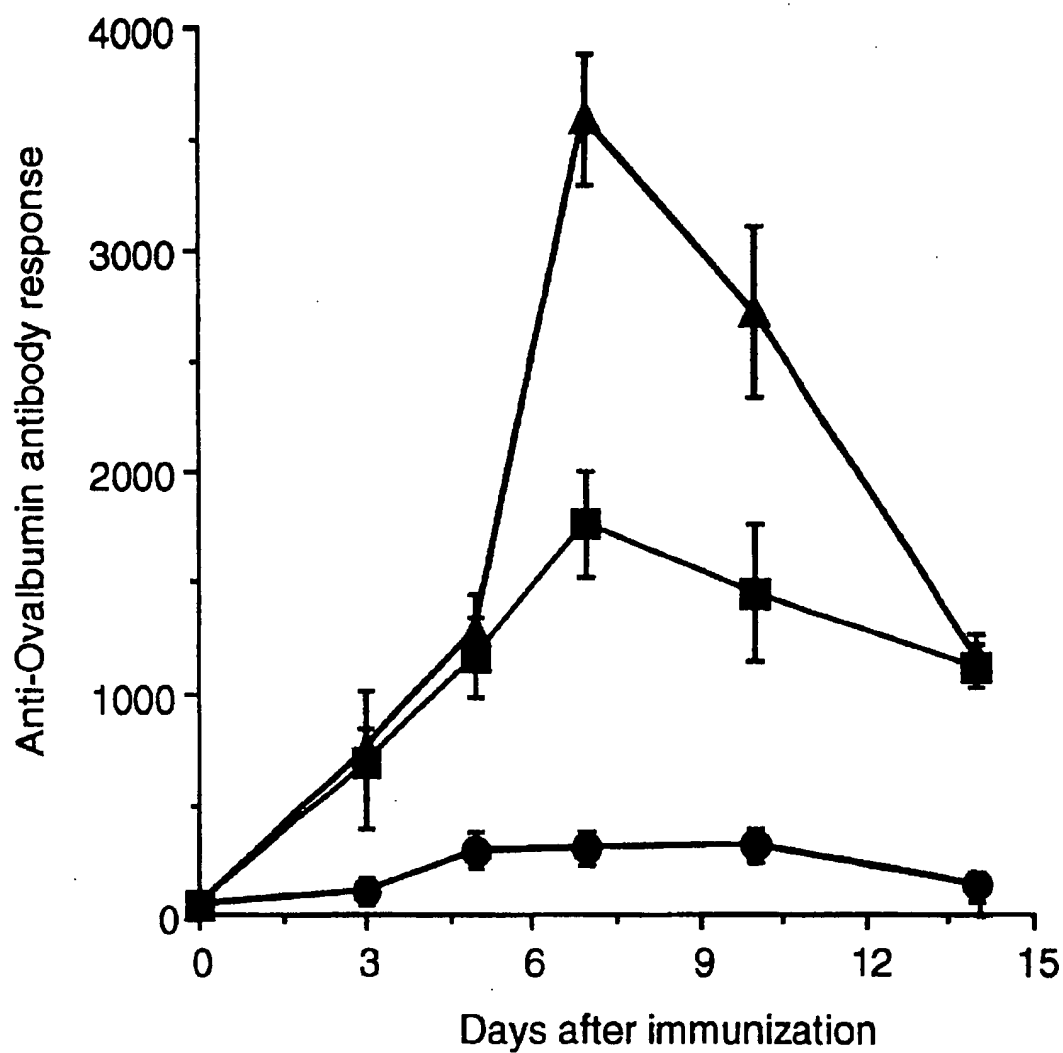


FIG.2G

**FIG.3A****FIG.3B****FIG.3C****FIG.3D****FIG.3E****FIG.3F**

**FIG.3G**

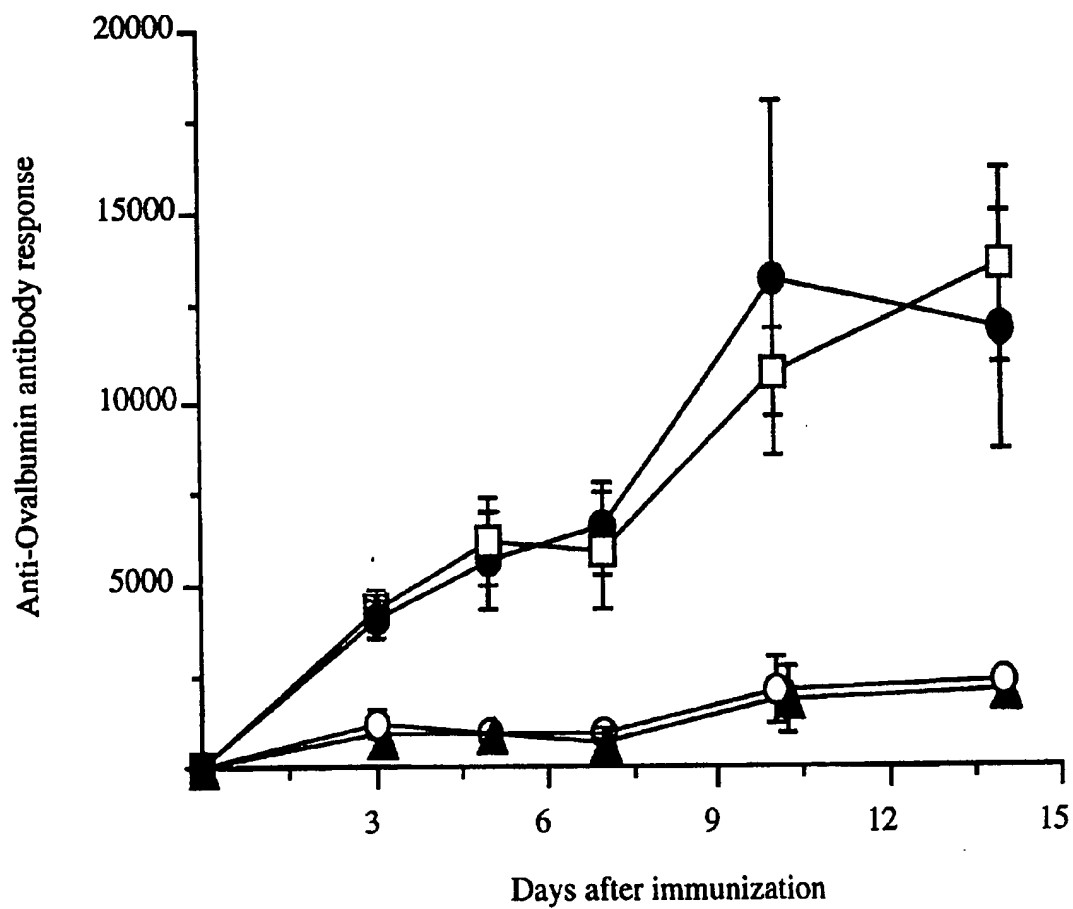


FIG.4

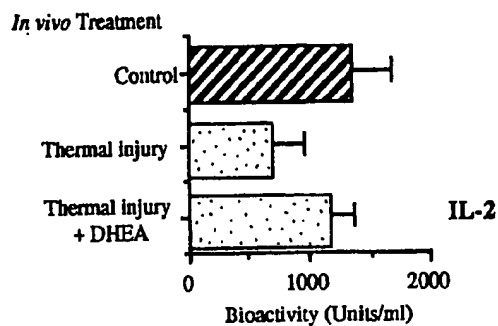


FIG.5A

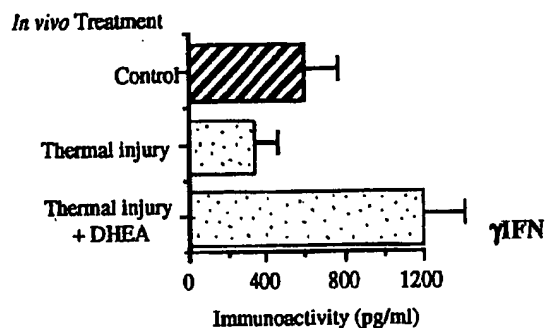


FIG.5B

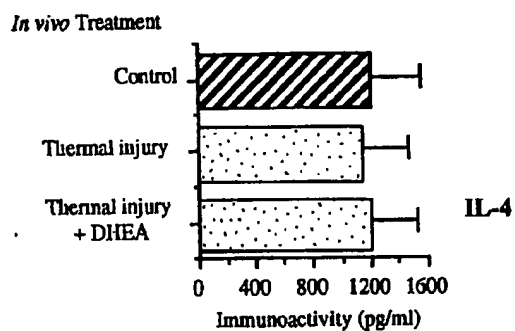


FIG.5C

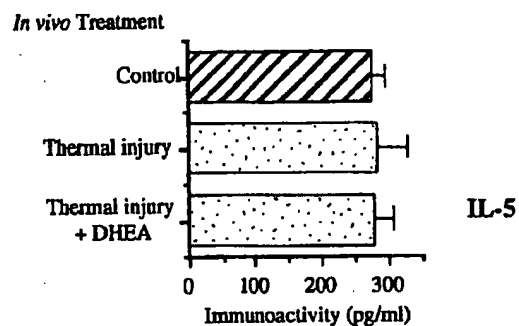


FIG.5D

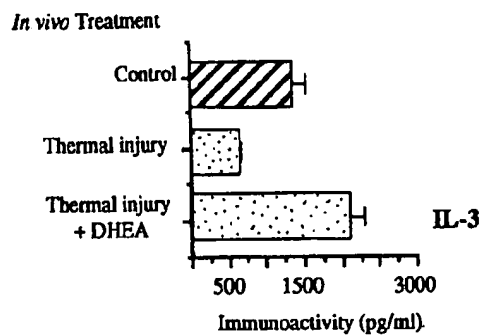


FIG.5E

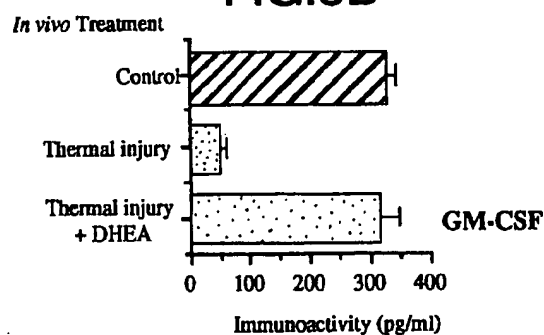
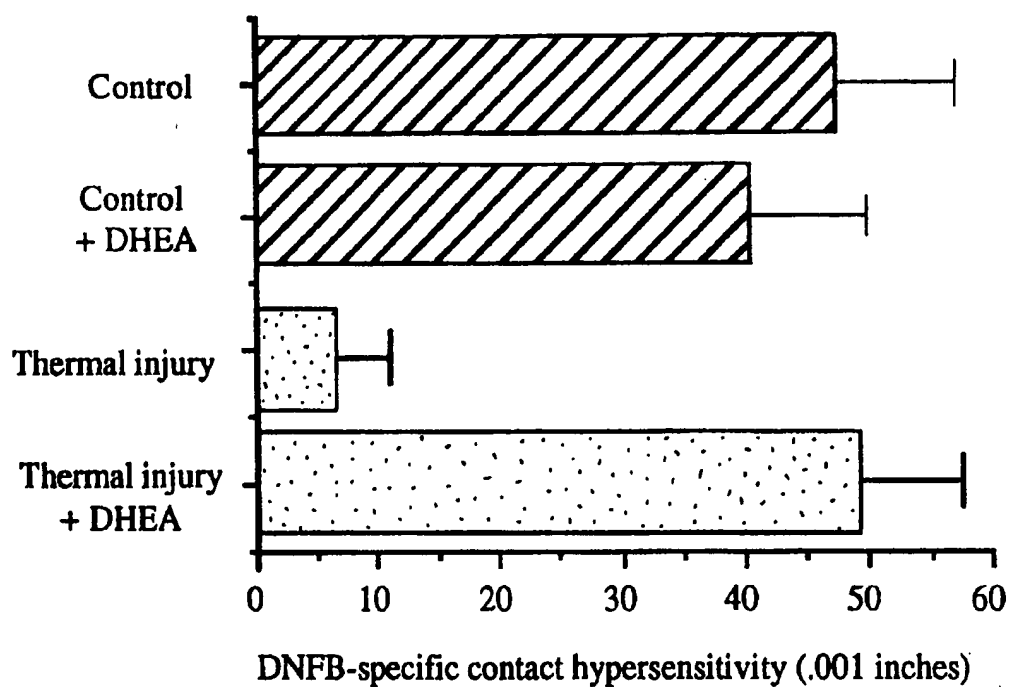


FIG.5F

*In vivo Treatment***FIG.6**

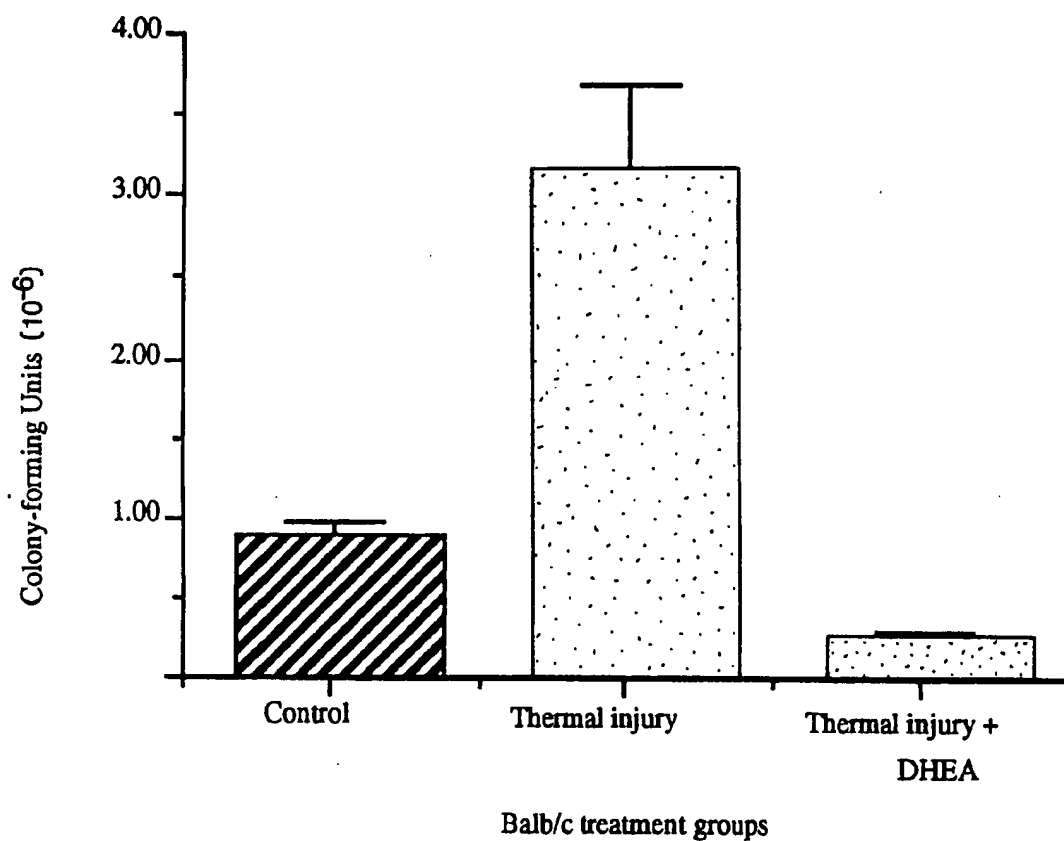


FIG.7

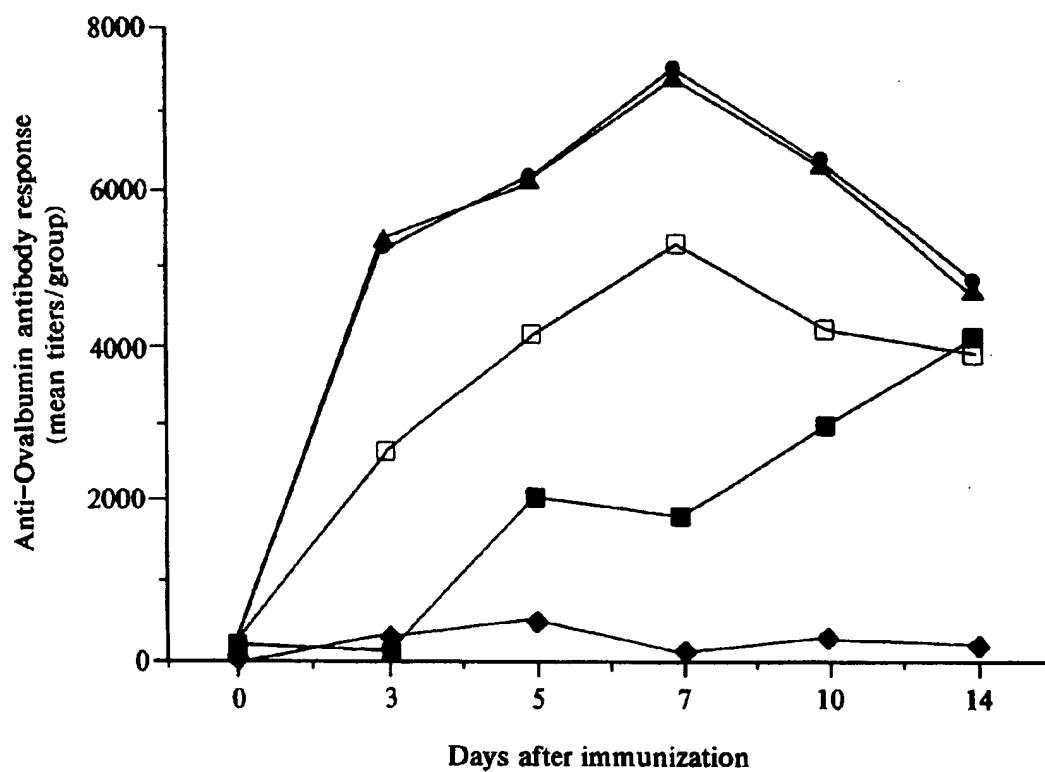


FIG.8

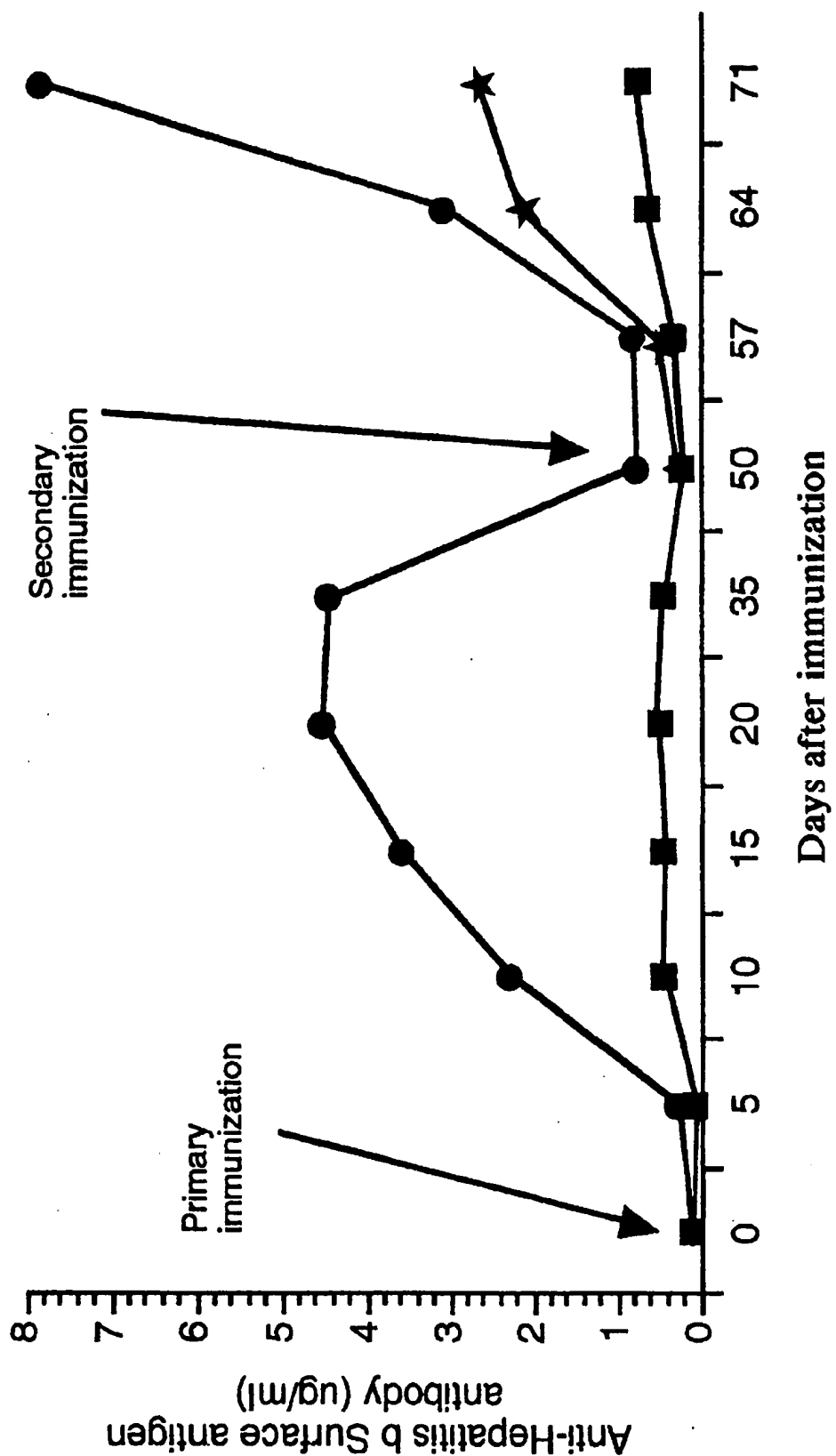


FIG. 9

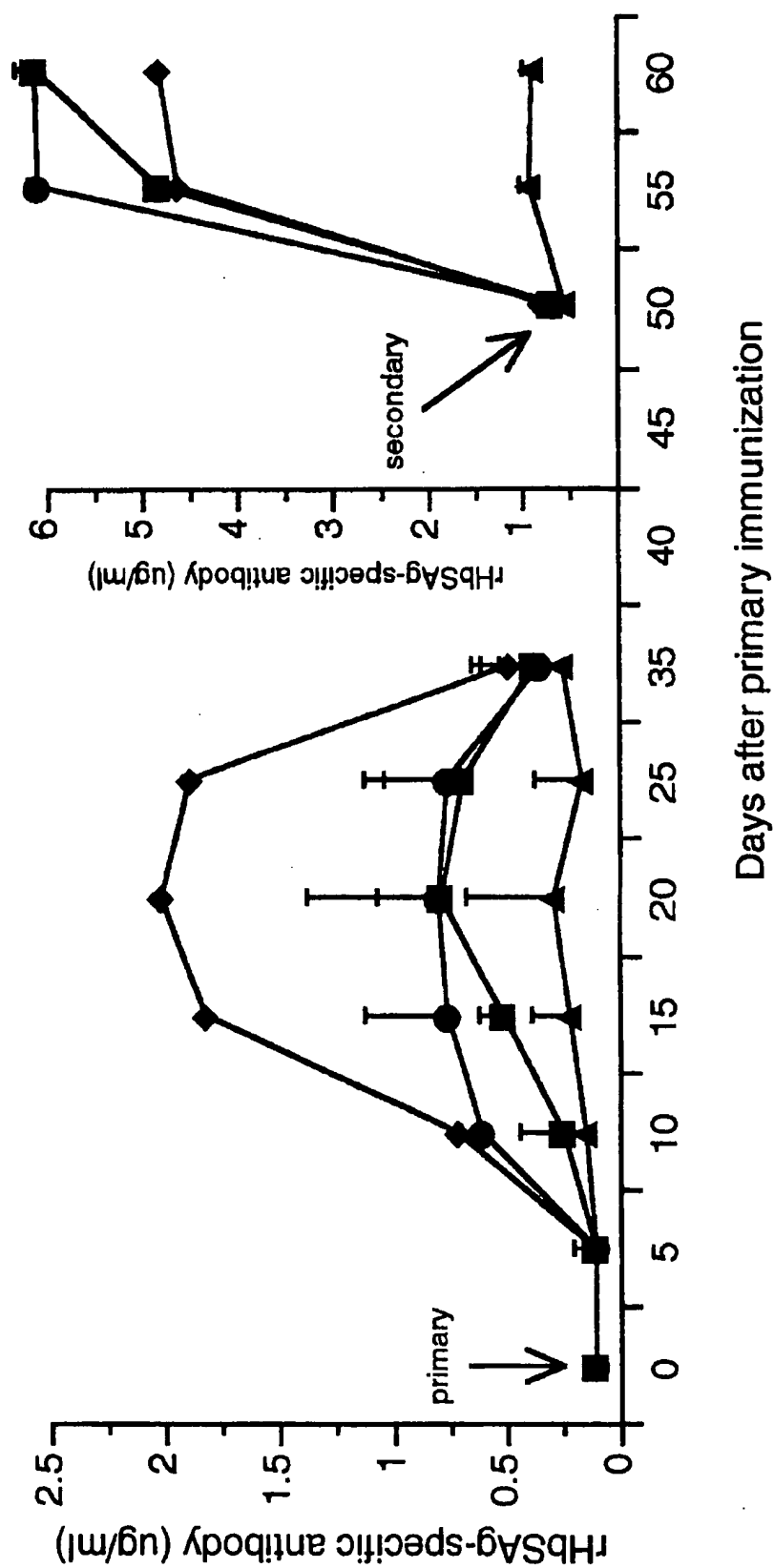


FIG. 10A

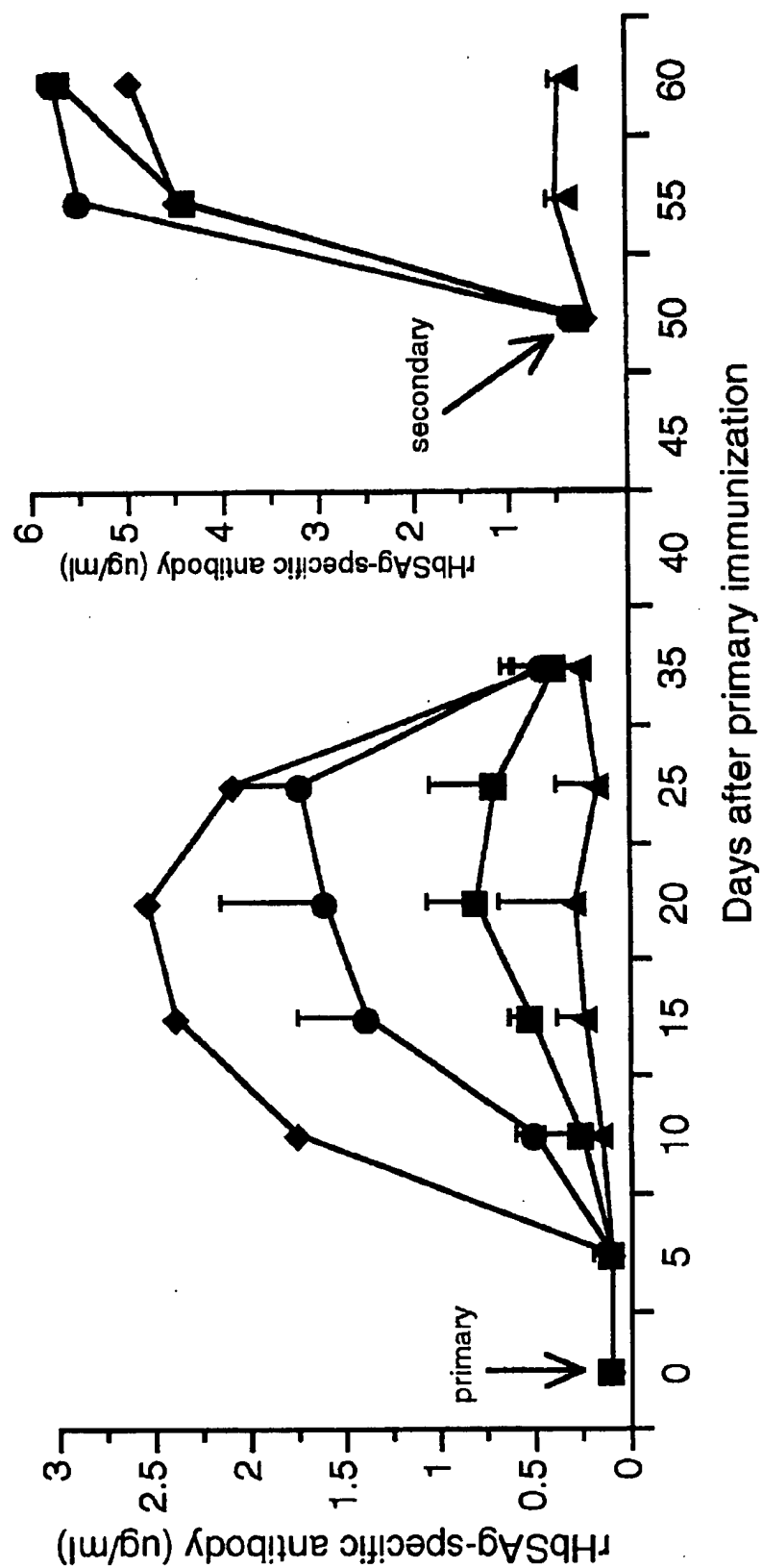
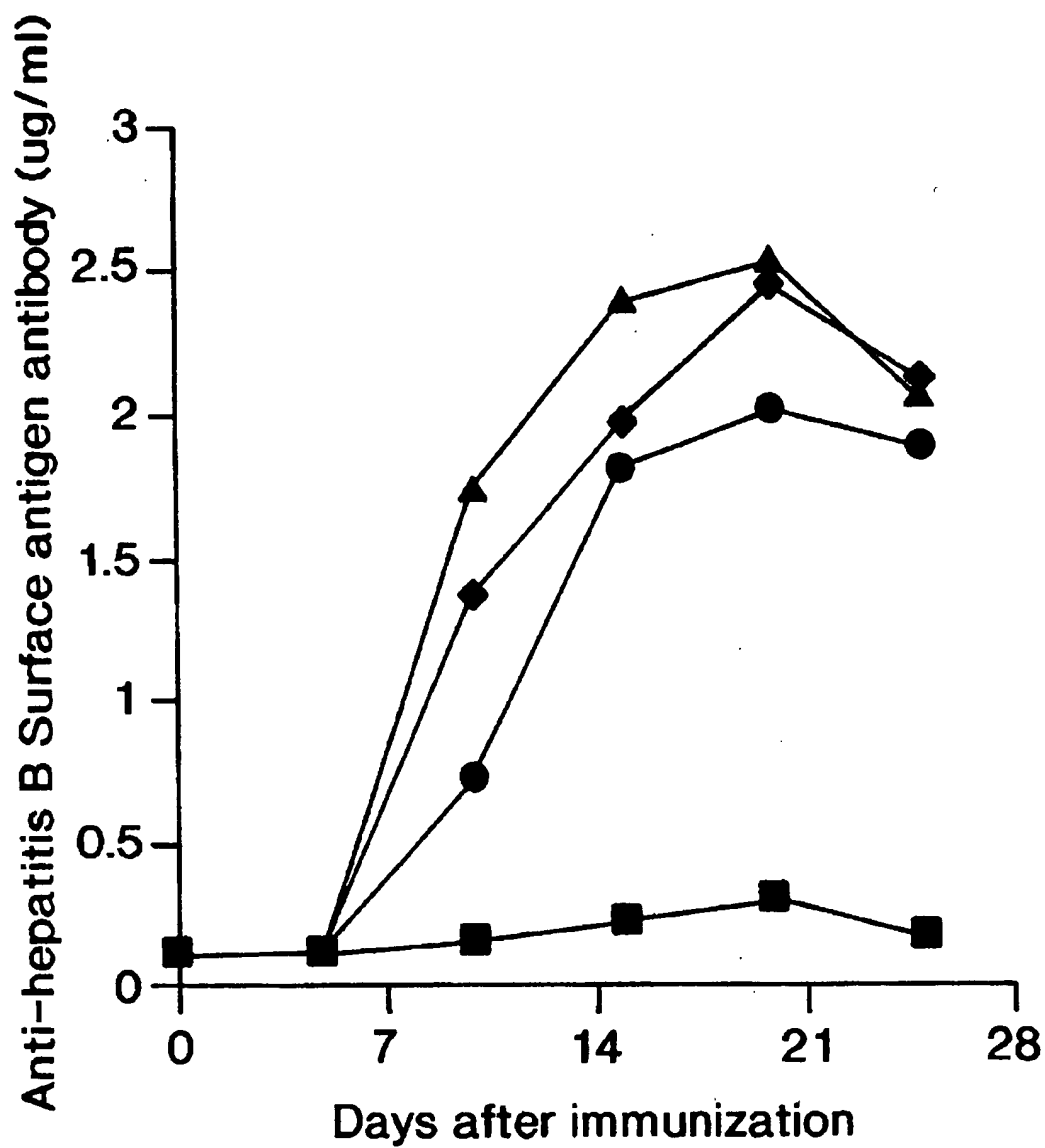


FIG. 10B

**FIG.11**

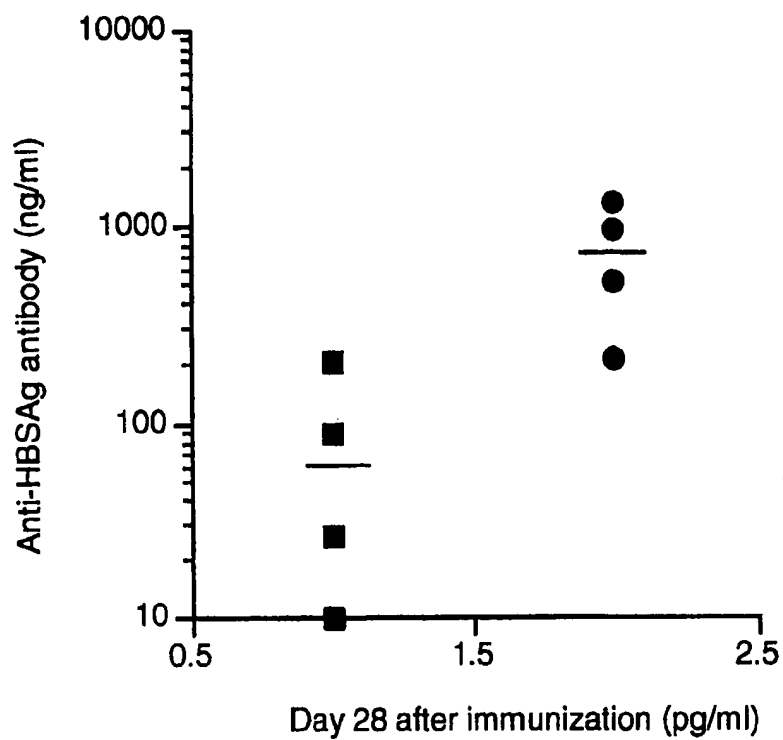


FIG. 12A

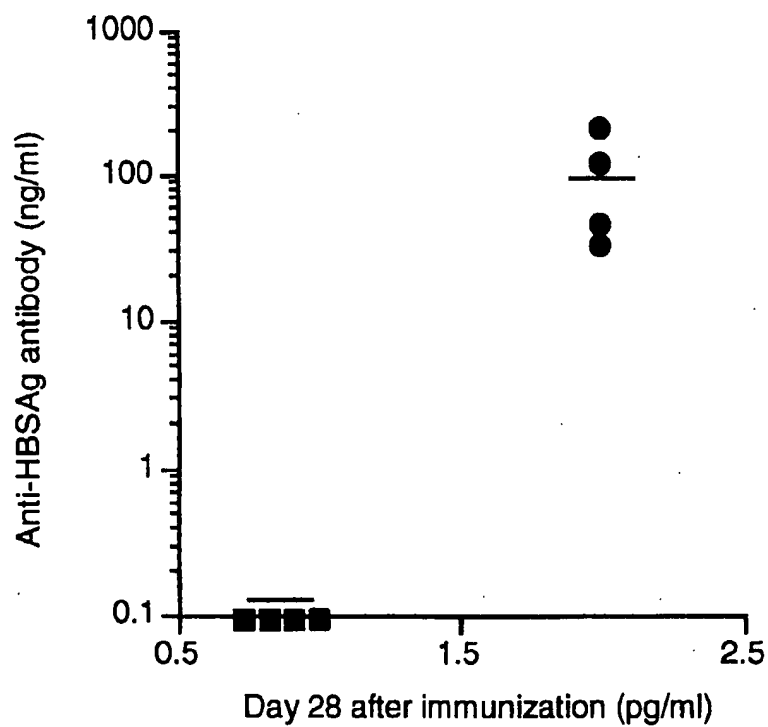
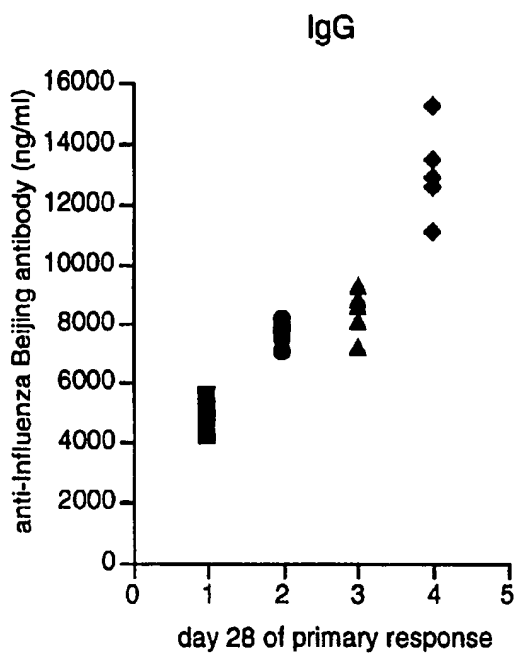
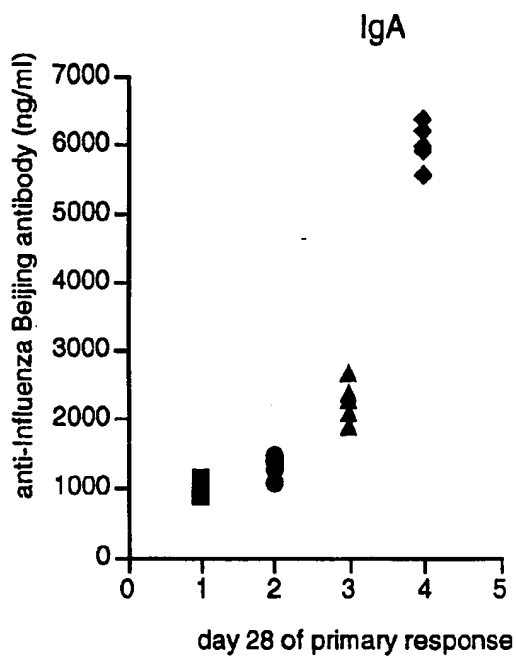


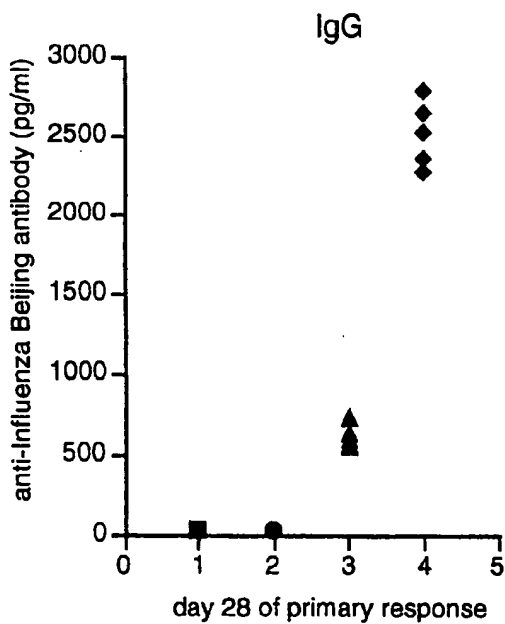
FIG. 12B



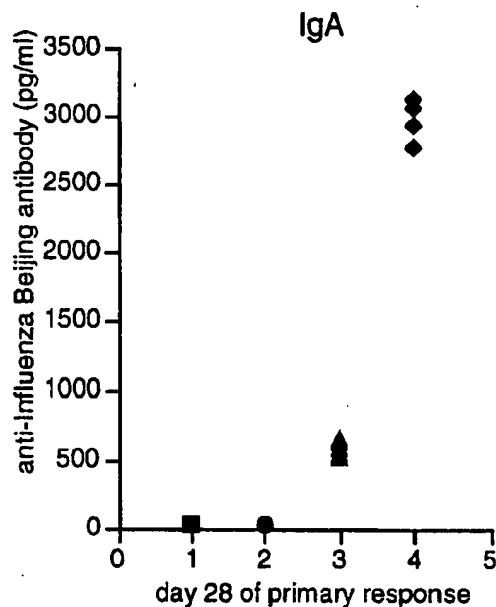
**FIG. 13A**



**FIG. 13B**



**FIG. 13C**



**FIG. 13D**

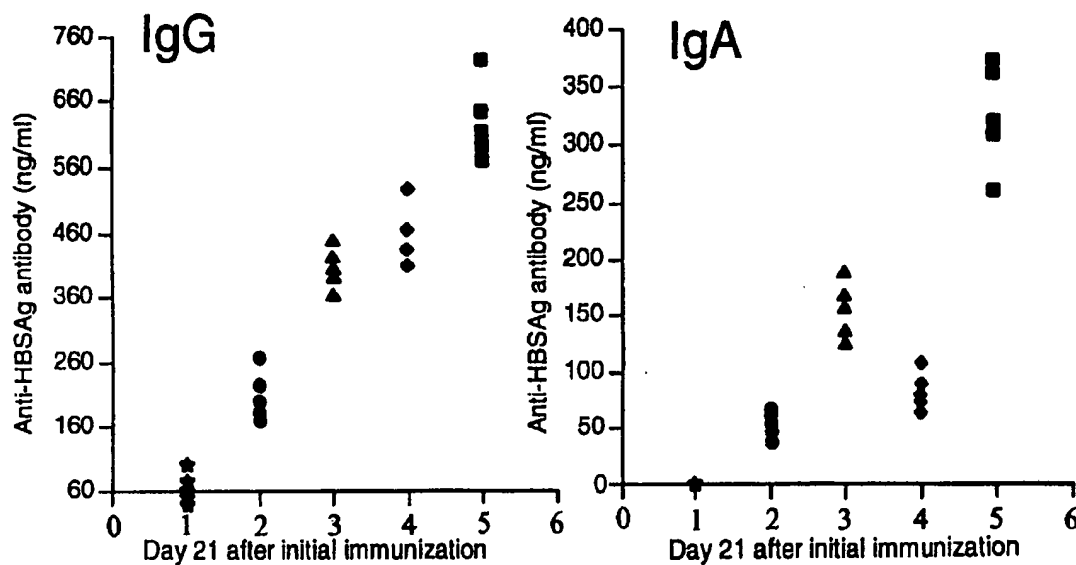


FIG. 14A

FIG. 14B

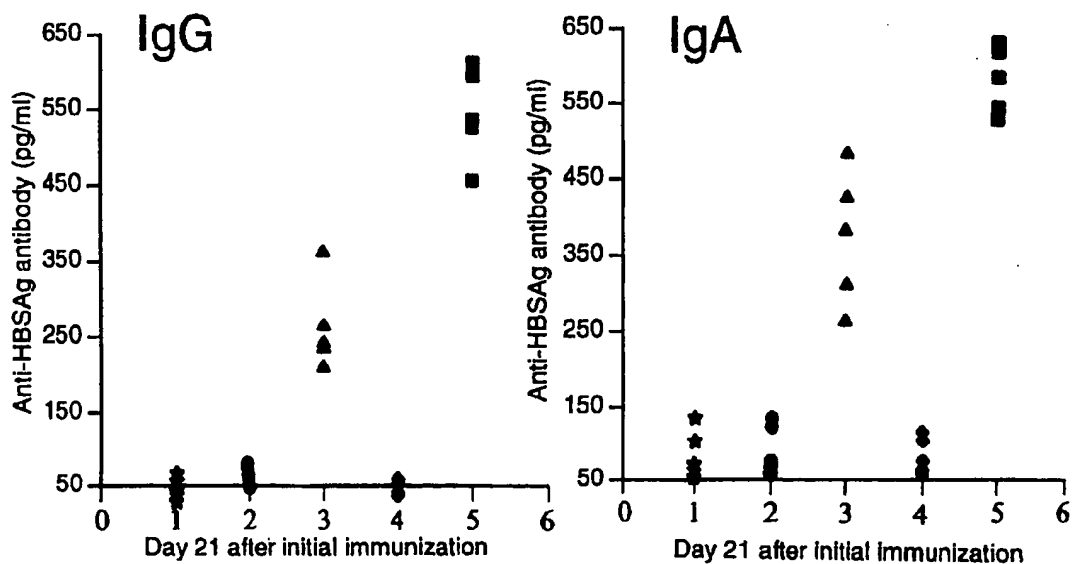
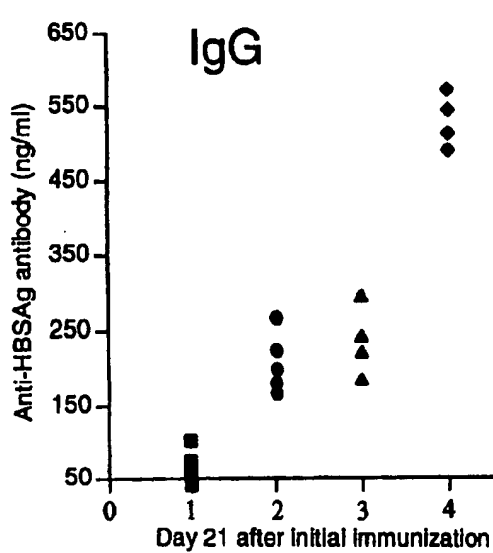
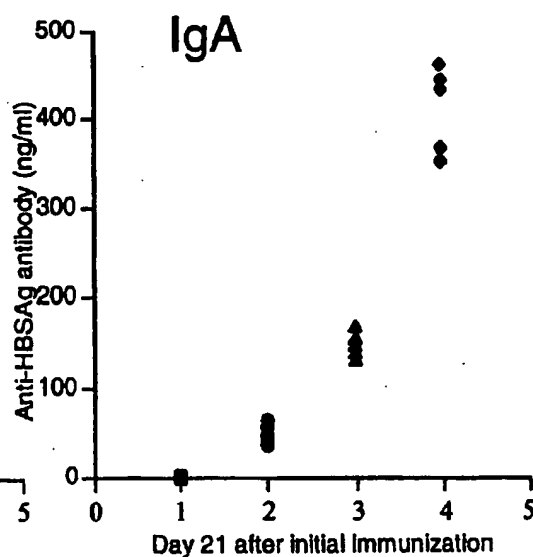


FIG. 14C

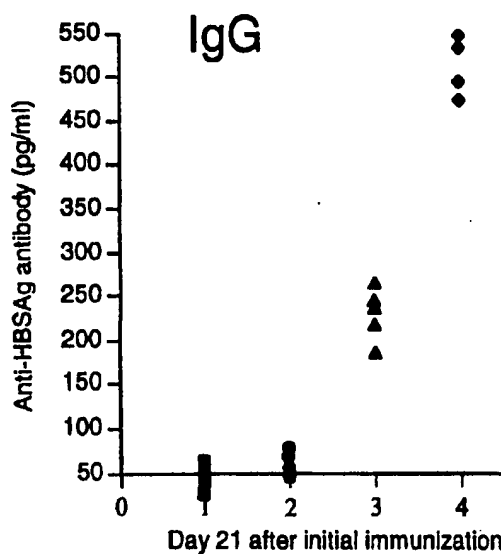
FIG. 14D



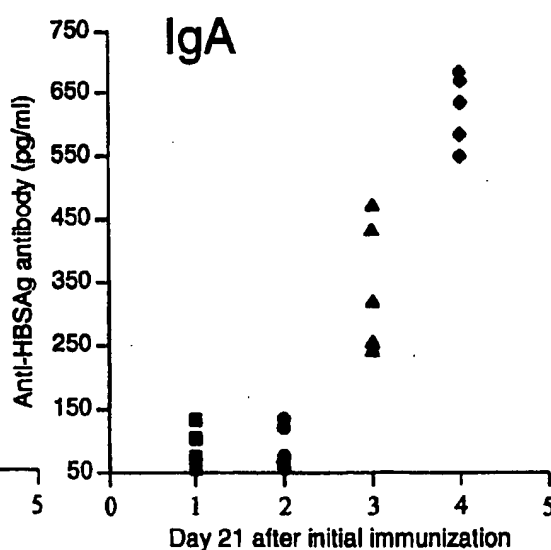
**FIG. 15A**



**FIG. 15B**



**FIG. 15C**



**FIG. 15D**

# VACCINE COMPOSITIONS AND METHOD FOR ENHANCING AN IMMUNE RESPONSE

## CROSS-REFERENCE TO RELATED APPLICATIONS

This Application is a continuation-in-part of U.S. application Ser. No. 08/013,972, filed Feb. 4, 1993 abandoned, and of U.S. application Ser. No. 07/779,499, filed Oct. 18, 1991, abandoned, which in turn is a continuation-in-part of U.S. application Ser. No. 07/412,270, filed Sep. 25, 1989, abandoned.

## TECHNICAL FIELD

The invention relates to vaccine compositions and methods of vaccination which provide for higher antibody titres in the vaccinated individuals. More specifically, the invention relates to vaccine compositions containing immune response augmenting agents, such as dehydroepiandrosterone (DHEA) and DHEA derivatives as immunomodulators in the vaccine compositions. The invention further relates to methods for enhancing a vaccine-induced humoral immune response.

## BACKGROUND OF THE INVENTION

It is known that lymphocytes exported from the thymus undergo a series of differentiation events which confer upon them the capacity to recognize and respond to specific peptide antigens presented appropriately in the context of self major histocompatibility complex (MHC) molecules. Mechanistically, thymic maturation is a complex process which includes an irreversible rearrangement of T cell receptor genes, the cell surface expression of these gene products as disulfide-linked heterodimers, positive and negative selection processes to provide appropriate restriction and avoidance of self-reactivity, and the synthesis and expression of CD4 or CD8 as accessory adhesion molecules. Microenvironmental influences within the thymus play an essential role in the fidelity of this process.

Subsequent to leaving the thymic microenvironment, mature T lymphocytes gain access to the recirculating T cell pool where they move freely via the blood between mucosal and nonmucosal lymphoid compartments in the mammalian host (Hamann et al. (1989). *Immunol. Rev.* 108: 19). T-lymphocyte expression of lymphoid tissue-specific homing receptors, which are complementary for vascular addressins on high endothelial venules present in Peyer's patches and peripheral lymph nodes, provide a biochemical means for selectivity to this recirculation process (id.). Non-activated lymphocytes can move freely between mucosal and nonmucosal lymphoid tissues due to the presence of both types of homing receptors on their plasma membranes (Pals et al. (1989). *Immunol. Rev.* 108: 111). Effector lymphocytes, and antigen-activated immunoblasts which are stimulated in a particular site in the body, however, exhibit a far more selective migratory behavior. These cells move primarily to tissues originally involved in antigen exposure and cellular activation (Hamann et al., supra; Pals et al., supra).

An immune response is initiated following T cell recognition of antigen peptides in the context of self MHC molecules, and generally takes place in one of the host's secondary lymphoid compartments. Cellular activation is triggered by the binding of antigen to the T cell receptor (TCR), forming an antigen/TCR complex which transduces the antigen-specific extracellular stimulation across the plasma membrane, and generates intracellular signals which

include the activation of protein kinase C and the increases in intracellular calcium. While signal transduction can lead to T cell unresponsiveness, positive signal transduction events trigger a series of additional biochemical processes. One consequence of this activation is the stimulated production of a number of biologically active molecules, which are collectively termed lymphokines. (See, Alcover et al. (1987). *Immunol. Rev.* 95: 5; Gelfand et al. (1987). *Immunol. Rev.* 95: 59).

Vaccines are preparations of antigenic material for administration to induce in the recipient an immunity to infection or intoxication by a given infecting agent. Vaccines may be prepared from viruses, rickettsiae, bacteria, protozoa and metazoa. Vaccines may be sterile suspensions of the killed organisms, of toxoids or other antigenic material derived from the organisms or recombinant sources, which can be administered by injection. Vaccines may be either simple vaccines prepared from one species of organism or a variety of organisms, or they may be mixed vaccines containing two or more simple vaccines. They are prepared in such a manner as not to destroy the antigenic material, although the methods of preparation vary, depending on the vaccine.

Vaccine adjuvants consist of agents that are included in the formulation that are used to enhance the ability of the antigenic material in a vaccine to induce the desired immune response, and with many poorly antigenic materials the success of vaccination depends on the presence of a suitable adjuvant in the vaccine. The adjuvant is sometimes conveniently incorporated in the vaccine before the latter is distributed into containers, although it may be provided in a separate container for mixing with the antigenic material when the vaccine is required for use in immunizing the recipient.

U.S. Pat. No. 4,698,221 discloses a vaccine which contains (a) an antigen, (b) a fat-soluble vitamin, such as Vitamin A, Vitamin D and/or Vitamin E, (c) a zinc compound, and (d) a selenium compound.

DHEA is a steroid hormone that has been extensively studied for many years. It has been reported to be involved in a wide variety of physiologic, immunologic, and pathologic conditions (for reviews, see Regelson et al. (1988). *Ann. N.Y. Acad. Sci.* 521: 260; Gordon et al. (1986). *Adv. Enzyme Reg.* 26: 355-382). Most endocrinologists believe that the primary function of DHEA is to serve as a precursor for the synthesis of testosterone and the estrogens by the gonads. Prior to its release into the bloodstream, the vast majority of newly synthesized DHEA becomes sulfated. The conjugated steroid DHEA-S is a secretory product of the adrenal gland in humans and certain primates. DHEA-S represents the major steroid hormone in the circulation of humans, and is converted to DHEA by the enzymatic activity of asteroid sulfatase.

Therapeutic uses for DHEA and certain analogs have been reported for diabetes, dry skin, ocular hypertension, obesity, and retroviral infections. Illustrative of these reports are the disclosures of U.S. Pat. No. 4,395,408, U.S. Pat. No. 4,518,595, U.S. Pat. No. 4,542,129, U.S. Pat. No. 4,617,299, U.S. Pat. No. 4,628,052, U.S. Pat. No. 4,666,898, published European Patent Application No. 0 133 995 A2, and published United Kingdom Patent Application No. GB 2 204 237 A.

## SUMMARY OF THE INVENTION

The invention relates to a vaccine which comprises an antigen and an immune response augmenting agent. The

immune response augmenting agent is capable of enhancing T cell lymphokine production. Suitable immune response augmenting agents include, but are not limited to, DHEA and DHEA-derivatives. Examples of DHEA derivatives include DHEA-sulfate (DHEA-S), 16 $\alpha$ -bromo-DHEA, 7-oxo-DHEA, 16 $\alpha$ -bromo-DHEA-S and 7-oxo-DHEA-S.

The invention also relates to a method for enhancing a vaccine-induced humoral immune response which comprises administering a vaccine which comprises an antigen and an immunomodulator. The immunomodulator may be an immune response augmenting agent, a lymphoid organ modifying agent or a mixture of the immune response augmenting agent and lymphoid organ modifying agent. Suitable lymphoid organ modifying agents include, but are not limited to, 1,25-dihydroxy Vitamin D<sub>3</sub>, biologically active Vitamin D<sub>3</sub> derivatives which are capable of activating the intracellular Vitamin D<sub>3</sub> receptor, all trans-retinoic acid, retinoic acid derivatives, retinol, retinol derivatives and glucocorticoid. Alternatively, the method for enhancing a vaccine-induced humoral immune response comprises separately administering the immunomodulator and a vaccine containing an antigen.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the effect of DHEA and 1,25(OH)<sub>2</sub>D<sub>3</sub> administered in vivo on lymphokine production by activated splenocytes.

FIG. 2A is a graph showing the result of DHEA-S supplementation on the capacity of T cells from aged mice to produce a IL-2.

FIG. 2B is a graph showing the result of DHEA-S supplementation on the capacity of T cells from aged mice to produce IL-4.

FIG. 2C is a graph showing the result of DHEA-S supplementation on the capacity of T cells from aged mice to produce IL-5.

FIG. 2D is a graph showing the result of DHEA-S supplementation on the capacity of T cells from aged mice to produce  $\gamma$ -IFN.

FIG. 2E is a graph showing the result of DHEA-S supplementation on the capacity of T cells from aged mice to produce IL-3.

FIG. 2F is a graph showing the result of DHEA-S supplementation on the capacity of T cells from aged mice to produce GM-CSF.

FIG. 2G is a graph showing the results of chronic DHEA-S supplementation on the humoral responsiveness of aged mice to an ovalbumin (OVA) challenge. The responsiveness is shown for mature adult mice ( $\square$ ), aged mice ( $\circ$ ), and aged mice with oral DHEA-S ( $\bullet$ ).

FIG. 3A is a graph showing the result of administration of a bolus of DHEA-S to aged mice on the ability of splenocytes from the treated mice to have restored IL-2 production.

FIG. 3B is a graph showing the results of administration of a bolus of DHEA-S to aged mice on the ability of splenocytes from the treated mice to have restored IL-4 production.

FIG. 3C is a graph showing the results of administration of a bolus of DHEA-S to aged mice on the ability of splenocytes from the treated mice to have restored IL-5 production.

FIG. 3D is a graph showing the results of administration of a bolus of DHEA-S to aged mice on the ability of

splenocytes from the treated mice to have restored  $\gamma$ -IFN production.

FIG. 3E is a graph showing the results of administration of a bolus of DHEA-S to aged mice on the ability of splenocytes from the treated mice to have restored IL-3 production.

FIG. 3F is a graph showing the results of administration of a bolus of DHEA-S to aged mice on the ability of splenocytes from the treated mice to have restored GM-CSF production.

FIG. 3G is a graph showing the results of administration of a bolus of DHEA-S to aged mice on the production of anti-ovalbumin antibodies by the treated mice. The antibody production is shown for mature adult mice ( $\square$ ), aged mice ( $\circ$ ), and aged mice with DHEA-S injection ( $\bullet$ ).

FIG. 4 is a graph showing the effect of topical DHEA application to aged mice on the production of anti-ovalbumin antibodies by the treated mice. The antibody production is shown for mature adult mice ( $\square$ ), aged mice ( $\circ$ ), aged mice with DHEA administration at the same site as the antigen ( $\bullet$ ), and aged mice with DHEA administration at the opposite site of the antigen ( $\blacktriangle$ ).

FIG. 5A is a graph showing the effect of DHEA treatment in vivo on the production of IL-2 by activated splenocytes from thermally injured and control mice.

FIG. 5B is a graph showing the effect of DHEA treatment in vivo on the production of  $\gamma$ -IFN by activated splenocytes from thermally injured and control mice.

FIG. 5C is a graph showing the effect of DHEA treatment in vivo on the production of IL-4 by activated splenocytes from thermally injured and control mice.

FIG. 5D is a graph showing the effect of DHEA treatment in vivo on the production of IL-5 by activated splenocytes from thermally injured and control mice.

FIG. 5E is a graph showing the effect of DHEA treatment in vivo on the production of IL-3 by activated splenocytes from thermally injured and control mice.

FIG. 5F is a graph showing the effect of DHEA treatment in vivo on the production of GM-CSF by activated splenocytes from thermally injured and control mice.

FIG. 6 is a graph showing the effect of DHEA treatment in vivo on contact hypersensitivity responses of thermally injured and control mice.

FIG. 7 is a graph showing the effect of DHEA treatment on resistance to *L. monocytogenes* in control and thermally injured C3H mice.

FIG. 8 is a graph showing the effect of topically applied DHEA, 16 $\alpha$ -bromo-DHEA and 16 $\alpha$ -chloro-DHEA on the anti-ovalbumin antibody response in aged mice. The antibody response is shown for young mice ( $\square$ ), old mice ( $\blacklozenge$ ), old mice with DHEA ( $\bullet$ ), old mice with 16 $\alpha$ -bromo-DHEA ( $\blacktriangle$ ), and old mice with 16 $\alpha$ -chloro-DHEA ( $\blacksquare$ ).

FIG. 9 shows the effect of topical administration of DHEA on vaccination of aged mice with recombinant Hepatitis B Surface Antigen (rHBSAg). The primary and secondary antibody responses are shown for aged mice without DHEA treatment ( $\blacksquare$ ), aged mice with DHEA treatment at time of primary immunization ( $\circ$ ), and aged mice with DHEA treatment at time of secondary immunization ( $\star$ ).

FIG. 10A shows that primary and secondary antibody responses in aged mice following vaccination with rHBSAg is enhanced by topically applied DHEA. The antibody responses are shown for mature mice without DHEA treatment ( $\blacksquare$ ), mature mice with DHEA treatment ( $\bullet$ ), aged

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mice without DHEA treatment (▲), and aged mice with DHEA treatment (◆).

FIG. 10B shows that primary and secondary antibody responses in aged mice following vaccination with rHBSAg is enhanced by incorporating DHEA as a component of the vaccine. The antibody responses are shown for mature mice without DHEA treatment (■), mature mice with DHEA treatment (●), aged mice without DHEA treatment (▲), and aged mice with DHEA treatment (◆).

FIG. 11 shows that the antibody response in aged mice, following vaccination with rHBSAg, is enhanced by treatment with DHEA or DHEA-S. The antibody responses are shown for aged mice without treatment (■), aged mice with topical DHEA treatment (●), aged mice with DHEA incorporated in the vaccine (▲), and aged mice with DHEA-S incorporated in the vaccine (◆).

FIG. 12A shows that DHEA administration enhances the efficiency of immunization with suboptimal doses of rHBSAg. The antibody responses are shown for mice without DHEA treatment (■) and with DHEA treatment (●), for vaccination with 0.5 µg rHBSAg/mouse.

FIG. 12B shows that DHEA administration enhances the efficiency of immunization with suboptimal doses of rHBSAg. The antibody responses are shown for mice without DHEA treatment (■) and with DHEA treatment (●), for vaccination with 0.1 µg rHBSAg/mouse.

FIGS. 13A and 13B show that serum (systemic) antibody response in mature mice following vaccination with Influenza-A Beijing strain is synergistically enhanced by treatment with DHEA and 1,25(OH)<sub>2</sub>D<sub>3</sub>. Antibody responses at Day 28 are shown for mice without treatment (■), mice treated with 2 µg DHEA in vaccine (●), mice treated with 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (▲), and mice treated with 2 µg DHEA and 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (◆).

FIGS. 13C and 13D shows that mucosal antibody response in mature mice following vaccination with Influenza-A Beijing strain is synergistically enhanced by treatment with DHEA and 1,25(OH)<sub>2</sub>D<sub>3</sub>. Antibody responses at Day 28 are shown for mice without treatment (■), mice treated with 2 µg DHEA in vaccine (●), mice treated with 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (▲), and mice treated with 2 µg DHEA and 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (◆).

FIGS. 14A and 14B shows that serum (systemic) antibody response in mature mice following vaccination with rHBSAg is synergistically enhanced by treatment with DHEA and 1,25(OH)<sub>2</sub>D<sub>3</sub>. Antibody responses at Day 21 are shown for non-immunized mice (★), mice without treatment (●), mice treated with 2 µg DHEA in vaccine (◆), mice treated with 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (▲), and mice treated with 2 µg DHEA and 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (■).

FIGS. 14C and 14D shows that mucosal antibody response in mature mice following vaccination with rHBSAg is synergistically enhanced by treatment with DHEA and 1,25(OH)<sub>2</sub>D<sub>3</sub>. Antibody responses at Day 21 are shown for non-immunized mice (★), mice without treatment (●), mice treated with 2 µg DHEA in vaccine (◆), mice treated with 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (▲), and mice treated with 2 µg DHEA and 0.1 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> in vaccine (■).

FIGS. 15A and 15B shows that serum (systemic) antibody response in mature mice following vaccination with rHBSAg is synergistically enhanced by treatment with DHEA and all trans-retinoic acid. Antibody responses at Day 21 are shown for non-immunized mice (■), mice without treatment (●), mice treated with 5.0 µg all trans-retinoic acid in vaccine (▲), and mice treated with 2 µg DHEA and 5.0 µg all trans-retinoic acid in vaccine (◆).

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FIGS. 15C and 15D shows that mucosal antibody response in mature mice following vaccination with rHBSAg is synergistically enhanced by treatment with DHEA and all trans-retinoic acid. Antibody responses at Day 21 are shown for non-immunized mice (■), mice without treatment (●), mice treated with 5.0 µg all trans-retinoic acid in vaccine (▲), and mice treated with 2 µg DHEA and 5.0 µg all trans-retinoic acid in vaccine (◆).

#### DETAILED DESCRIPTION OF THE INVENTION

The most important function of the immune system is to provide its host with protection against diseases. To carry out these tasks, a large and diverse array of effector mechanisms have evolved, the majority of which exhibit antigen specificity. Each individual effector mechanism possesses a degree of uniqueness with respect to its ability to influence the rate of progression, to detoxify, or to promote the elimination of microbial pathogens or tumor cells. Such a diversity in available mechanisms is absolutely essential, since no single effector response can effectively deal with all forms of pathogenic insults. Furthermore, to protect normal function of the various non-lymphoid organ systems and tissues of the body requires careful selection, activation, and compartmentalization of the most appropriate types of immune effector mechanisms. Equally important is the simultaneous capacity to down-regulate the development of other types of responses. Immunologic effector responses must, therefore, be both effective and practical, and at the same time be appropriately regulated anatomically to reduce the risk of pathologic consequences.

The non-lymphoid tissues and organs of the body, which work collectively to sustain the life of the host, must also be capable of providing regulatory information to cells of the immune system. This information, mediated through the activities of inflammation-induced tissue cytokines, prostaglandins and other types of biological response modifiers, becomes integrated into the complex equation to control the mechanisms which regulate effector response selection.

T cells, through their capacity to produce a number of lymphokines in response to activation, play a central role in guiding the development of immune effector responses. Mechanisms which operate to control the synthesis and secretion of these pleiotropic biologic response modifiers, therefore, directly influence the quantitative and qualitative nature of immunity. The lymphokines and cytokines provide important information, not only to cells of the immune system, but also to cells of the other tissue and organ systems. For this information to be meaningful, it is essential that lymphokine production remains tightly controlled at the levels of both cellular source and duration. Autocrine and paracrine effects by lymphokines and cytokines should be the norm, since only a few species are capable of working effectively when provided via endocrine routes. These essential anatomic restrictions, therefore, cannot be adequately provided by bolus injection of recombinant lymphokines and/or cytokines, and may explain the limited success associated with this form of therapy.

The vast majority of the T cells in the peripheral circulation are known to reside within the recirculating T cell pool. These cells continuously enter and exit secondary lymphoid organs throughout the body, maintaining residence within any particular site for only finite periods of time. Over the lifespan of any individual mature T cell, therefore, it has probably taken up temporary residence in

most of a host's secondary lymphoid organs. T cell recirculation provides the immune system with a means for clonally-restricted T cells to provide a level of surveillance over all of the tissue and organ systems.

It is universally accepted that most T cells acquire their specificity for antigen, and for a self-MHC-restricting element, during processes which occur at the time of their ontogeny within the thymus. However, the extent to which intrathymic maturation confers genetic restrictions upon individual T cells that regulate their potential for immunologic involvement has not been delineated.

A general concept which explains the results in the Examples, but which is not intended to limit the invention, is that the genetic programs of resting recirculating T cells are continuously being altered by extrinsic environmental influences. The steroid hormones, either presented systemically in their active forms (e.g., as glucocorticosteroids (GCS)), or provided to T cells only within discrete microenvironments as a consequence of end-organ metabolism (e.g., as DHEA, DHT or  $1,25(\text{OH})_2\text{D}_3$ ), perform important roles in this process. The basal regulation of the immune system at the level of the T cell requires the continual presence of the needed substrates (prohormones). The anatomic compartmentalization of functional potential for T cells, therefore, would be dependent on the cellular source of the steroid metabolizing enzymes able to convert the steroid hormone substrates to their bioactive species. It has been shown that macrophages can contain each of these enzymes.

More specifically, DHEA-S can be naturally converted to DHEA in the peripheral lymph nodes of animals with normal immune function. The DHEA produced then influences the T lymphocytes within the lymph node and exerts controlling influences on their ability to respond when activated. This provides a means to regulate the potential of T cells by fluctuating the degree to which a particular steroid hormone exists within a particular tissue. Old individuals and/or stressed individuals, including humans, have a reduced capacity to produce DHEA-S, resulting in altered T-cell responsiveness. The invention in its various embodiments restores the metabolite produced from DHEA-S in the anatomic compartment in which appropriate T-cell responsiveness is required for normal immune responses to T-cell-dependent antigens.

The invention relates to a vaccine which comprises an antigen and an immune response augmenting agent. The immune response augmenting agent is capable of enhancing T cell lymphokine production. Suitable immune response augmenting agents include, but are not limited to, DHEA and DHEA-derivatives. Examples of DHEA derivatives include DHEA-sulfate (DHEA-S),  $16\alpha$ -bromo-DHEA, 7-oxo-DHEA,  $16\alpha$ -bromo-DHEA-S and  $7\alpha$ -oxo-DHEA-S.

The invention also relates to a method for enhancing a vaccine-induced humoral immune response which comprises administering a vaccine which comprises an antigen and an immunomodulator. The immunomodulator may be an immune response augmenting agent, a lymphoid organ modifying agent or a mixture of the immune response augmenting agent and lymphoid organ modifying agent. Suitable lymphoid organ modifying agents include, but are not limited to,  $1,25$ -dihydroxy Vitamin  $\text{D}_3$ , biologically active Vitamin  $\text{D}_3$  derivatives which are capable of activating the intracellular Vitamin  $\text{D}_3$  receptor, all trans-retinoic acid, retinoic acid derivatives, retinol, retinol derivatives and glucocorticoid. Alternatively, the method for enhancing a vaccine-induced humoral immune response comprises separately administering the immunomodulator and a vaccine containing an antigen.

As used herein, the term "immunomodulator" refers to an agent which is able to modulate an immune response. An example of such modulation is an enhancement of antibody production.

The term "individual" refers to a vertebrate and preferably to a member of a species which naturally produces DHEA and DHEA-S and possesses DHEA-S sulfatase activity, and includes, but is not limited to domestic animals, sports animals and primates, including humans.

The term "effective amount" of an immunomodulator refers to an amount of an immunomodulator sufficient to enhance a vaccine-induced humoral immune response. An effective amount of an immunomodulator, if injected, can be in the range of about 0.1–1,000  $\mu\text{g}$ , preferably 5–500  $\mu\text{g}$ , for a human subject, or in the range of about 0.01–10.0  $\mu\text{g}/\text{Kg}$  body weight of the subject animal. This amount may vary to some degree depending on the mode of administration, but will be in the same general range. If more than one immunomodulator is used, each one may be present in these amounts or the total amount may fall within this range.

An effective amount of an antigen may be an amount capable of eliciting a demonstrable humoral immune response in the absence of an immunomodulator. For many antigens, this is in the range of about 5–100  $\mu\text{g}$  for a human subject. Since the vaccines of the invention utilize an immunomodulator which enhances the humoral immune response, it may be possible to utilize less antigen, e.g., about 1–5  $\mu\text{g}$  for a human subject.

The exact effective amount necessary will vary from subject to subject, depending on the species, age and general condition of the subject, the severity of the condition being treated, the mode of administration, etc. Thus, it is not possible to specify an exact effective amount. However, the appropriate effective amount may be determined by one of ordinary skill in the art using only routine experimentation or prior knowledge in the vaccine art.

"Treatment" refers to the administration to an individual of a composition which yields a protective immune response, and includes prophylaxis and/or therapy.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a secretory, humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen."

The specific antigen can be a protein, a polysaccharide, a lipopolysaccharide or a lipopeptide; or it can be a combination of any of these. Particularly, the specific antigen can include a native protein or protein fragment, or a synthetic protein or protein fragment or peptide; it can include glycoprotein, glycopeptide, lipoprotein, lipopeptide, nucleoprotein, nucleopeptide; it can include a peptide-peptide conjugate; or it can include a recombinant nucleic acid expression product. Examples of antigens include, among others, those that are capable of eliciting an immune response against vital or bacterial hepatitis, influenza, diphtheria, tetanus, pertussis, measles, mumps, rubella, polio, pneumococcus, herpes, respiratory syncytial virus, hemophilus influenza type b, chlamydia, varicellazoster virus or rabies.

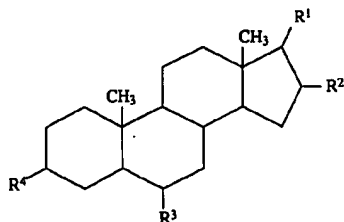
An "immunological response" to a composition or vaccine comprised of an antigen is the development in the host of a cellular- and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

By "vaccine composition" or "vaccine" is meant an agent used to stimulate the immune system of an individual so that current harm is alleviated, or protection against future harm is provided.

"Immunization" refers to the process of inducing a continuing protective level of antibody and/or cellular immune response which is directed against an antigen to which the organism has been previously exposed.

A "pharmacologic dose" is one which gives a desired physiological effect.

"DHEA" or "DHEA-derivative", as used herein, refer to compounds having the formula



wherein

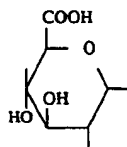
$R^1$  is  $=O$  or  $OH$ ;

$R^2$  is  $H$  or halogen;

$R^3$  is  $H$  with a 5-6 double bond or  $=O$ ;

$R^4$  is  $OR^5$ ;

$R^5$  is  $H$ ,  $SO_2OM$ ,  $PO_2OM$  or a glucuronide group of the formula



$M$  is  $H$ ,  $Na$ ,  $K$  or  $-CH_2-CH(OH)-CH_2-O-CO-R^6$ ; and  
 $O-CO-R^7$

$R^6$  and  $R^7$  may be the same or different and may be a straight or branched  $C_{1-14}$  alkyl.

"Lymphoid organ modifying agent," as used herein, means a biological response modifier that is capable, when administered to a vertebrate animal in vivo at a peripheral site, of altering the microenvironment of a peripheral lymphoid organ that drains from the administration site, such that activated lymphocytes and macrophages residing within the lymphoid organ exhibit a pattern of cytokines more typical of the microenvironment of a lymphoid organ of the mucosal lymphoid compartment. Particularly, a pattern of cytokines more typical of a mucosal lymphoid organ is characterized by relatively enhanced production of one or more of active TGF- $\beta$ , IL-4, IL-5, and IL-10, and relatively decreased production (or at least no relatively enhanced production) of one or more of IL-2 and IFN- $\gamma$ . In preferred embodiments, the lymphoid organ modifying agent includes a biological response modifier that can, or a combination of biological response modifiers that together can, when administered to a peripheral lymph organ, result in the lymphoid organ exhibiting a pattern of cytokines more typical of a mucosal lymphoid organ. Lymphoid organ modifying agents are described in U.S. application Ser. No. 08/013,972 and U.S. application Ser. No. 08/123,844, U.S. Pat. No. 5,518,725, entitled "Vaccine Compositions and

Method for Induction of Mucosal Immune Response via Systemic Vaccination," filed concurrently herewith, to latter of which is incorporated herein by reference. By way of example, it can be a substance known to enhance or facilitate production of active TGF- $\beta$ , such as, for example, all trans-retinoic acid, retinoic acid derivative, retinol or retinol derivatives, or it can be 1,25(OH) $_2$ D $_3$  or its biologically active derivatives, or possibly glucocorticoids. Biologically active Vitamin D $_3$  derivatives are those derivatives capable of activating the intracellular Vitamin D $_3$  receptor. Preferably, the lymphoid organ modifying agent is administered epicutaneously at a peripheral anatomical site (such as, for human subjects, for example, the arm or buttocks or leg); and the specific antigen is administered to the same anatomical site, or to a site known to drain into the same lymphoid organ that receives drainage from the site of administration of the lymphoid organ modifying agent. In one mode of administration, the lymphoid organ modifying agent can be combined with the specific antigen and immune response augmenting agent for simultaneous administration at the same site. The lymphoid organ modifying agent, the specific antigen and immune response augmenting agent, or all of them, can be administered by injection.

An "immune response augmenting agent", as used herein, means an agent that is capable, when administered to a vertebrate animal in vivo, of restoring T cell responsiveness to T cell dependent antigens characteristic of normal immune responses to such antigens. Immune response augmenting agents are capable of enhancing T cell lymphokine production, particularly IL-2, IL-3, IFN- $\gamma$  and GM-CSF. By way of example, the immune response augmenting agent can be a substance, such as DHEA or DHEA-derivative, which enhances T cell lymphokine production.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., ANIMAL CELL CULTURE (R. I. Freshney, Ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987); and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, Volumes I-IV, (D. M. Weir and C. C. Blackwell, Eds., 1986). All patents, patent applications and publications mentioned herein, both supra and infra, are incorporated herein by reference.

The simultaneous enhancement or maximization of the production of more than one T cell lymphokine may be achieved by exposing the T cell lymphocyte to more than one immunomodulator prior to cellular activation. The exposure to more than one immunomodulator can be simultaneous or sequential. The concentration of each of the immunomodulators should be balanced to achieve the desired enhancing effects on the vaccine-induced humoral immune response.

Evidence derived from experimental and clinical observations indicates that immunologic reactions elicited to either simple or complex antigens often manifest as a balanced heterogeneous blend of both cellular and humoral components, with the fractional contribution of any individual type of effector mechanism often dominating the overall response. This level of heterogeneity is essential to the development of a protective immune response. Alterations to this natural balance, whether caused by genetic or physiologic changes associated with age or stress or trauma,

can lead to a depressed capacity to elicit protective immune responses, and might also lead to immunologic responses having pathologic consequences.

Pharmaceutical compositions made up of formulations comprised of the immunomodulators and suitable for administration by subcutaneous, epicutaneous, topical, intramuscular or intradermal routes and the like may be prepared by one of ordinary skill in the art. See, for example, *Remington's Pharmaceutical Sciences*, 17th Ed. (1985, Mack Publishing Co., Easton, Pa.). For example, the pharmaceutical composition containing the immunomodulator may also contain a physiologically acceptable carrier that is non-toxic to the treated animal and is compatible with the steroid. Suitable pharmaceutical carriers include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations.

The pharmaceutical composition containing the immunomodulator is used as a vaccine adjuvant to enhance a vaccine-induced humoral immune response. When the individuals are immunized with an immunizing agent, administration of the immunomodulator may be prior to, contemporaneously with, or after the vaccination. Typical methods of administering the immunomodulator include mixing the immunomodulator with the immunizing agent (antigen) in a vaccine or topically applying the immunomodulator to skin sites which drain into the same lymph nodes as the antigen of the vaccine. This latter method is preferably used with individuals who are immunologically deficient due to low levels of DHEA-S and/or DHEA and in whom one wishes to augment the immune response, for example, the aged or neonates or individuals who are therapeutically immunosuppressed.

One or more immunomodulators may be used to enhance the vaccine-induced immune response. They may be administered sequentially or contemporaneously. It is preferred to administer them contemporaneously and in a single vehicle. It has been discovered that a synergistic enhancing effect is achieved when an immune response augmenting agent, as defined herein, is combined with a lymphoid organ modifying agent, as defined herein, and used as the immunomodulator. It is preferred to include the two agents in the vaccine, although either or both may also be topically applied. In general, an effective amount of immunomodulator may be about 0.1–1,000 µg, preferably 5–500 µg, for a human subject. An effective amount of an immune response augmenting agent may be about 10–1,000 µg, preferably 20–200 µg, for a human subject if administered by injection. If the immune response augmenting agent is administered orally, the amount may range from about 10–100 mg/day, preferably 20–50 mg/day, for a human subject, or in the range of about 0.5–5 mg/Kg/day for an animal subject. An effective amount of a lymphoid organ modifying agent may be about 0.1–500 µg, preferably 0.5–250 µg, for a human subject.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

#### EXAMPLE 1

The Effect of DHEA and 1,25(OH)<sub>2</sub>D<sub>3</sub> in vivo on IL-2 and IL-4 Production in vitro

C3H mice received implants of biodegradable DHEA or 1,25(OH)<sub>2</sub>D<sub>3</sub> pellets designed to deliver steroid at a rate of

10.4 and 2.6 µg/hour, respectively. Three days after implantation, both the steroid treated groups of mice and a normal control group were immunized in the hind footpads with 100 µg OVA in CFA. Ten days after immunization, the draining lymph nodes and spleens from all groups were prepared for culture. Lymph node cells were stimulated with 100 µg OVA. Culture supernatants were assayed for IL-2 and IL-4 activity after 24 hours using the HT-2 bioassay. The results are shown in FIG. 1. From the Figure, it is seen that DHEA administration caused an approximately four-fold increase in IL-2 production, and no stimulation of IL-4 production. In contrast, 1,25(OH)<sub>2</sub>D<sub>3</sub> administration caused an approximately eight-fold increase in IL-4 production, but did not stimulate IL-2 production.

Similar alterations in the ability of antigen-activated T cells to produce IL-2 and IL-4 were observed when the immune response augmenting agent was mixed with the immunizing antigen, or was topically applied to skin sites above the site of vaccination.

#### EXAMPLE 2

Preservation of Normal Potential to Produce T-cell Lymphokines and Generate Humoral Immune Responses by Supplementation with DHEA Sulfate

Circulating levels of DHEA sulfate decline markedly with advancing age in humans and other mammals. As shown in U.S. application Ser. No. 07/779,499, abandoned, direct treatment of T cells from aged or normal murine donors with DHEA prior to activation in vitro augmented their capacity to produce IL-2. In contrast, DHEA-S, the prohormone form of DHEA found principally in the circulation, was shown to have no direct effect on T-cell production of this lymphokine. When DHEA-S was administered to normal mature adults in vivo, it enhanced the potential for IL-2 production by T cells isolated from lymphoid organs having the greatest DHEA-S activity. The most active lymphoid organs are those having anatomic positions downstream of nonmucosal tissues. This Example demonstrates that DHEA-S supplementation in vivo can influence the age-related changes in lymphokine production and humoral immune responses.

Groups of adult BALB/c mice, between 35 and 39 weeks of age, were separated into two groups. One group was provided with 100 µg/ml DHEA-S in their drinking water. The hormone was offered ad libitum to these animals. The other group was left untreated. Mice were maintained on oral DHEA-S supplementation until age 114 weeks, when they were sacrificed and their spleens individually analyzed for the capacity to produce lymphokines following anti-CD3ε activation. The DHEA-S treated and untreated mice were evaluated by comparing their responses to the lymphokine profile produced by similarly activated splenocytes from mature adult mice (13 weeks of age).

More specifically, splenocytes were prepared from the following groups of BALB/c mice; 2 mature adult (13 weeks), 2 aged (114 weeks), and 2 aged (114 weeks) receiving 100 µg/ml DHEA-S in their drinking water for the previous 61 weeks. 1×10<sup>7</sup> splenocytes were cultured under serum-free conditions in triplicate and activated with 1 µg/ml CD3ε. Culture supernatants were analyzed for the level of IL-2 by a quantitative bioassay, and for IL-4, IL-5, γ-IFN, IL-3 and GM-CSF by capture ELISA.

FIGS. 2A–2F are graphs showing the results of DHEA-S supplementation on the capacity of T cells to produce a variety of lymphokines. In FIGS. 2A–2F, bars

represent the mean  $\pm$ SD for the value of each lymphokine presented. It is seen from FIGS. 2A-2F that DHEA-S supplementation, administered prior to the onset of age-induced decline in immunocompetence, is accompanied by the preservation of normal lymphokine production and development of normal humoral immune responses. DHEA-S supplementation was not only able to preserve normal levels of IL-2, IL-3, and GM-CSF production by activated T cells, but was also able to prevent age-related increase in  $\gamma$ -IFN, IL-4, and IL-5 production seen in the cell supernatants from untreated aged donors. The results of this study demonstrate that a striking correlation exists between the age-related decline in endogenous DHEA production (plus its metabolites) and the age-associated alterations in T-cell production of lymphokines.

The effect of DHEA-S supplementation on T cell function was also performed using BALB/c, C57BL/6 and C3H/HeN strains of mice. In each test of this experimental approach, lymphokine production by T cells from the treated aged donors had been preserved.

In order to examine the effect of DHEA-S supplementation on the ability of old animals to mount immunologic responses to challenge with foreign protein antigens, the following procedure was used. Groups of 5 mature adult mice (13 weeks), 5 aged mice (114 weeks), and 5 aged mice (114 weeks) provided with chronic DHEA-S supplementation (100  $\mu$ g/ml DHEA-S in their drinking water for the previous 61 weeks, initiated at 8 months of age), were footpad-immunized with ovalbumin. The immunization was with 100  $\mu$ g ovalbumin in a 25  $\mu$ l volume of Maalox, administered in the hind footpads. All animals were bled on Days 0, 3, 5, 7, 10, and 14 post-immunization, and individual serum samples analyzed for ovalbumin specific antibody titres by quantitative ELISA, using ovalbumin for capture and HRPO-coupled, goat anti-murine Ig detecting antibodies with specificity for IgM and IgG subclasses. Each ELISA assay was controlled with sera known to be positive or negative for anti-ovalbumin activity. The titre is the inverse of the antibody dilution equal to the half-maximal point on the titration curve. The results of the study, shown in the graph in FIG. 2G, demonstrate that old animals provided with chronic DHEA-S supplementation remain fully capable of rapidly mounting a significant humoral immune response to ovalbumin immunization, with kinetics, titres, and isotype profiles that are almost identical to mature adult controls. As expected, the untreated aged mice responded poorly to a similar antigen challenge, producing predominantly IgM.

### EXAMPLE 3

#### DHEA-S Administration to Aged Mice Can Reverse Age-Associated Changes in T-cell Lymphokine Production and Their Depressed Humoral Immune Responses to Protein Antigens

As shown in U.S. application Ser. No. 07/779,499, abandoned, a direct exposure of lymphocytes from aged donors to DHEA in vitro immediately altered the pattern of lymphokines produced following activation. In addition, it has been found that nonmucosal tissue-draining lymphoid organs possess a far greater amount of DHEA sulfatase activity than mucosal tissue-draining lymphoid organs. These findings led to the hypothesis that DHEA may be serving as an effector of positional information for lymphocytes residing in certain lymphoid compartments. Any changes in immune function caused by the depressed pro-

duction of substrate DHEA-S might, therefore, be reversible if DHEA-S is reintroduced in situ. This was examined in the following studies.

Splenocytes were isolated from equal-sized groups of mature adult mice (25 weeks), aged mice (120 weeks), and aged mice (120 weeks) given a subcutaneous injection of DHEA-S (100  $\mu$ g in 100  $\mu$ l propylene glycol) 24 hours previously.  $1 \times 10^7$  splenocytes were cultured under serum-free conditions in triplicate and activated with 1  $\mu$ g/ml anti-CD3 $\epsilon$ . Twenty-four hours later, culture supernatants from individual cell cultures were analyzed for the level of IL-2 by a quantitative bioassay, and for IL-4, IL-5,  $\gamma$ -IFN, IL-3 and GM-CSF by capture ELISA. The results, shown in FIGS. 3A-3F, demonstrate that acute replacement therapy to aged mice with DHEA-S restores near-normal patterns of T-cell lymphokines within one day of treatment. These results strongly show that lymphoid cells from old animals exhibit no intrinsic defects. Rather, some of the best documented functional changes to the immune system which accompany aging are due to the reduced capacity to produce DHEA-S.

A representative study, showing that administration of a bolus of DHEA-S to aged BALB/c mice restored the capacity of the mice to develop humoral responses, is shown in FIG. 3B. In the study, groups of 5 mature adult (25 weeks), 5 aged (120 weeks), and 5 aged (120 weeks) mice receiving 100  $\mu$ g DHEA-S in 100  $\mu$ l propylene glycol by subcutaneous injection the previous 24 hours were immunized with 100  $\mu$ g ovalbumin in a 25  $\mu$ l volume of Maalox, administered in the hind footpads. Sera from individual mice were collected on Days 0, 3, 5, 7, 10 and 14 following primary immunization. The titre of anti-ovalbumin antibody was assessed by ELISA using ovalbumin for capture and HRPO-coupled, goat anti-murine Ig detecting antibodies with specificity for IgM and IgG subclasses. Each ELISA assay was controlled with sera known to be positive or negative for anti-ovalbumin activity.

The results in FIG. 3G show that old animals provided With DHEA-S only 24 hours prior to immunization with a foreign protein antigen responded even better than normal mature adults in the production of antibody.

This method of reversing age-related decline in humoral responses has been evaluated twice using BALB/c mice and once with C3H/HeN strains of mice. Similar enhancements in antibody production were achieved in all groups of DHEA-S treated, aged groups of mice.

The results described in U.S. application Ser. No. 07/779,499, abandoned, and discussed above demonstrate that some of the age-associated changes in immune function are extrinsic in cause, and are mediated by the loss in endogenous production of an essential regulatory steroid prohormone.

### EXAMPLE 4

#### Topical Application of DHEA to Aged Animals Facilitates Changes in the Draining Lymph Node Microenvironment that are Conducive to Successful Immunization

Groups of 5 mature adult (13 weeks) and 10 aged BALB/c mice (114 weeks) were used in the study. All of the aged BALB/c mice received a topical application of 10  $\mu$ g DHEA in 3.5  $\mu$ l 95% ethanol to the right hind footpad, 3 hours prior to immunization with 100  $\mu$ g ovalbumin in a 25  $\mu$ l volume of Maalox. Five of the aged mice were immunized in the right hind footpad (at a site identical to the steroid application), and the other 5 immunized in the left hind footpad (site

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opposite to the steroid application). Sera from individual mice were collected on Days 0, 3, 5, 7, 10 and 14 following primary immunization. The titre of anti-ovalbumin antibody was assessed by ELISA using ovalbumin for capture and HRPO-coupled, goat anti-murine Ig detecting antibodies with specificity for IgM and IgG subclasses. Each ELISA assay was controlled with sera known to be positive or negative for anti-ovalbumin activity.

The results, shown in FIG. 4, establish that a topical application of DHEA, prior to immunization through the same skin site, provided the aged animals with the ability to generate completely normal humoral immune responses. The untreated group of aged animals, and aged animals provided with topical DHEA on the footpads opposite the site of immunization, responded quite poorly to immunization, with minimal antibody being observed.

The results of reversing the age-related decline in humoral responses has been repeated with BALB/c mice, and with C3H/HeN strain of mice. The results establish that the pronounced lymphoid organ-specific changes in the types of lymphokines produced by T cells from aged animals given topical DHEA, can be paralleled by an equally dramatic enhancement in ability to generate potent humoral immune responses to challenge with a foreign antigen protein.

#### EXAMPLE 5

##### Treatment in vivo of Thermally-Injured Mice with DHEA Preserves Normal Immune Function

U.S. application Ser. No. 07/779,499, abandoned, demonstrates that thermal injury to mice caused a depression in the capacity of activated T-cells to secrete IL-2, IFN- $\gamma$ , IL-3 and GM-CSF, and that this depression could be prevented with DHEA treatment. The following study illustrates that direct administration of DHEA to mice shortly after thermal injury influences their levels of immunocompetence.

Groups of 12 thermally-injured and 6 control BALB/c mice were established as described above. After subjecting the mice to a 20% total body surface area (TBSA) scald burn, six of the thermally-injured mice were given a subcutaneous injection of 100  $\mu$ g DHEA in a propylene glycol carrier. All remaining animals received the carrier alone. Five days later, all surviving mice were sacrificed and their splenocytes were individually prepared for culture, and activated with anti-CD3 $\epsilon$  to induce lymphokine secretion. Culture supernatants were collected 24 hours after activation and evaluated for lymphokine content, as described in U.S. application Ser. No. 07/779,499, abandoned. The results of the study are presented in FIGS. 5A-5F, where the bars represent mean  $\pm$ SD for each value. As seen in FIGS. 5A-5F, DHEA directly influences IL-2,  $\gamma$ -IFN, IL-3, and GM-CSF production by T cells isolated from thermally-injured mice. The administration of a single bolus injection of DHEA (100  $\mu$ g) 1 hour after thermal injury was sufficient to preserve for at least 5 days a normal capacity by their lymphocytes to produce IL-2,  $\gamma$ -IFN, IL-3, and GM-CSF following activation. No significant changes from normal were observed in the levels of IL-4 and IL-5 made by activated lymphoid cells from these animals.

The effect of DHEA treatment in vivo on the animals' development of cellular immune responses was examined. Parallel groups of thermally-injured and control mice were given either 100  $\mu$ g DHEA in propylene glycol carrier or the carrier alone 1 hour post-burn. These animals were contact-sensitized 5 days later by administration of DNFB on the

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abdomen. Challenge doses of DNFB to the right footpads were applied 4 days later. The differences in thickness between the right (challenged) and the left (unchallenged) footpads were used to quantitate the contact hypersensitivity responses. The results are shown in FIG. 6; the bars represent mean  $\pm$ SD for each group of mice. As shown in FIG. 6, the intensity of contact hypersensitivity responses elicited by thermally-injured mice is markedly depressed, as compared to controls. Administration of DHEA to thermally-injured mice was found to completely preserve the ability of these animals to develop contact hypersensitivity responses of normal intensity.

These studies demonstrate that DHEA treatment post-burn is an effective therapy for preserving the capacity of T-lymphocytes from thermally-injured animals to produce normal quantities of a number of lymphokines, especially those that are essential for development of cellular immune responses. This finding is supported by the demonstration in U.S. application Ser. No. 07/779,499, abandoned, that DHEA-treated thermally-injured mice also retain their capacity to develop normal contact hypersensitivity responses.

#### EXAMPLE 6

##### DHEA Treatment in vivo Promotes Resistance to Infection by *L. monocytogenes* in Thermally-Injured Mice

This study addresses the utility of DHEA therapy post-burn in preserving resistance to a bacterial infection. C3H/HeN strain mice are inherently resistant to infection by the gram-positive intracellular pathogen, *L. monocytogenes*. However, thermal injury results in increased susceptibility to this pathogen. Therefore, a switch from "resistant" to a more "susceptible" phenotype provides a model system to evaluate the effect of DHEA on preserving the "resistant" phenotype in thermally-injured animals.

Normal (control) and thermally-injured mice were prepared as described above. Half of the thermally-injured mice received a single bolus injection of 100  $\mu$ g DHEA subcutaneously within 1 hour after thermal injury. Three days later, all mice were infected with  $2 \times 10^6$  viable *L. monocytogenes* organisms, and 3 days after infection the mice were sacrificed and homogenates of individual spleens were prepared. The number of colonies of *L. monocytogenes* per spleen were evaluated using standard methodology, and scored. The results are presented graphically in FIG. 7, where the bars represent means  $\pm$ SEM for each treatment group. The results indicate that thermal injury enhances the susceptibility of the C3H strain mice to infection by *L. monocytogenes*. Of consequence, DHEA treatment of burned animals not only preserves the resistant phenotype, but surprisingly, the level of resistance to infection is actually enhanced by DHEA treatment over that observed in the control group.

#### EXAMPLE 7

##### The Effect of 16 $\alpha$ -bromo-DHEA and 16 $\alpha$ -chloro-DHEA on the Depressed Humoral Responses of Aged Mice to Protein Antigens

The effectiveness of the congeners of DHEA, 16 $\alpha$ -bromo-DHEA and 16 $\alpha$ -chloro-DHEA on age-associated changes in the humoral immune response is demonstrated in the following study. Groups of mature (young) and aged (old) mice were treated by topical administration of 10  $\mu$ g of DHEA,

16 $\alpha$ -bromo-DHEA, or 16 $\alpha$ -chloro-DHEA. Three hours subsequent to treatment, the treated and control animals were subjected to an ovalbumin (OVA) challenge as described in the Examples above, and the anti-ovalbumin antibody response was measured at Days 0, 3, 5, 7, 10, and 14 after immunization. The results, shown in FIG. 8, indicate that 16 $\alpha$ -bromo-DHEA is as effective as DHEA in restoring humoral immune responsiveness. The 16 $\alpha$ -chloro-DHEA yielded a lower, but significant, effect on antibody production.

#### EXAMPLE 8

##### Topical Administration of DHEA Enhances Antibody Production with Vaccines to Hepatitis B Surface Antigen

Groups of five mice [(C3H/HeNxC57BL/6)F1], 72 weeks of age, were immunized with 1.5  $\mu$ g Hepatitis B surface antigen (HBsAg) in aluminum hydroxide (273  $\mu$ g/ml; alum). Five of the mice were pre-treated with 10  $\mu$ g DHEA by topical administration 3 hours prior to immunization. Sera from individual mice were collected during the primary response. Prior to a secondary immunization on Day 50, five of the aged control mice were treated with 10  $\mu$ g DHEA by topical administration. All of the mice were then given a secondary immunization of 1.5  $\mu$ g HBsAg in alum and sera were collected weekly. Specific response to the vaccination was measured by quantitative ELISA. Purified rHBsAg diluted in 0.5M Tris-HCl, pH 9.6, at a concentration of 2  $\mu$ g/ml was dispensed into 96-well plates. Following incubation for a minimum of two hours at room temperature, or overnight at 4° C., all plates were blocked with PBS-0.05% Tween 20/1.0% bovine serum albumin (BSA) for an additional two-hour incubation at room temperature. Prior to adding the test samples, the plates were washed free of blocking buffer using three washes of distilled water and one wash with PBS/0.05% Tween 20. Individual samples were first diluted in PBS 0.05% Tween 20/1.0% BSA, and 100  $\mu$ l was then dispensed into appropriate wells of the antigen-coated plates. Included on each plate was an Ig standard: a series of two-fold dilutions of either purified IgG (all subclasses) or IgA (reference standards). The reference Ig were captured by goat anti-murine Ig which is known to bind all murine Ig isotypes. These plates were incubated at room temperature overnight, followed by 3 $\times$  wash in distilled water and one wash in PBS/0.05% Tween 20. The detection antibody (HRP-lined goat anti-mouse Ig specific for IgG and IgA) was diluted in PBS/Tween/ 10% normal goat serum at a dilution recommended by the manufacturer. After a final incubation and wash series, the ELISA was developed using ABTS-substrate. O.D. readings were recorded at 405 nm using a Vmax 96-well microtest plate spectrophotometer (Molecular Devices, Menlo Park, Calif.). A simple linear regression analysis of the Ig titration generated a reference curve for calculating the amount of specific antibody contained in the test samples. These data are reported as ng/ml  $\pm$ SEM. The results are shown in FIG. 9, and confirm the findings shown in Examples 2 and 3. FIG. 9 demonstrates that topical administration of DHEA provides an enhancement in the immune response for vaccination with HBsAg, as seen by serum anti-HBsAg antibodies.

Similar effects were seen with the topical administration of 16- $\alpha$ -Br-DHEA.

#### EXAMPLE 9

##### Administration of DHEA Topically Prior to Vaccination or as a Component of the Vaccine Enhances Antibody Production

Aged mice [(C3HxBL/6)F1], approximately 24 months old, were given a topical application of 10  $\mu$ g DHEA three

hours prior to vaccination with a recombinant Hepatitis B surface antigen (rHBsAg). Alternatively, 10  $\mu$ g DHEA was incorporated into the vaccine/alum mixture prior to immunization. Untreated aged mice, and untreated mature adult mice [(C3HxBL/6)F1], 26 weeks of age, were administered the ethanol vehicle without DHEA. All of the test mice were vaccinated with rHBsAg (1.0  $\mu$ g) in 25  $\mu$ l alum (273  $\mu$ g/ml) through the anatomic site of DHEA or ethanol application. Serum samples were collected from individual mice at multiple times during the primary response. A secondary antigen exposure was given to all mice 50 days after the primary immunization. Recall responses were stimulated without any additional treatment with DHEA. Serum samples were then collected 5 and 10 days following secondary antigen exposure. Individual serum samples were evaluated by quantitative ELISA as described above to determine the amount of HBsAg-specific antibody. The mean and SEM antibody responses at each interval are shown in FIGS. 10A and 10B. The results show that the serum antibody response to rHBsAg was enhanced in aged mice when the mice were treated prior to vaccination with topical DHEA (FIG. 10A) or were treated with DHEA incorporated in the vaccine (FIG. 10B).

#### EXAMPLE 10

##### Administration of DHEA or DHEA-S Topically or as a Component of the Vaccine Enhanced Antibody Production

Groups of sex- and age-matched mice [(C3HxC57BL/6)F1], greater than 24 months of age, were immunized subcutaneously with rHBsAg (1.0  $\mu$ g in 25  $\mu$ l alum (273  $\mu$ g/ml)). Animals were given a topical administration of 10  $\mu$ g DHEA three hours prior to vaccination. Alternatively, 10  $\mu$ g DHEA or DHEA-S was incorporated into the vaccine/alum mixture prior to immunization. Untreated aged mice were administered the ethanol vehicle without DHEA. Serum samples were collected from individual mice at multiple times and were evaluated by quantitative ELISA as described above, to determine the amount of HBsAg-specific antibody. The mean antibody response is shown in FIG. 11. The results show that the serum antibody response was enhanced in aged mice when the mice were treated prior to vaccination with topical DHEA (●) or were treated with DHEA (▲) or DHEA-S (◆) incorporated in the vaccine.

#### EXAMPLE 11

##### Administration of DHEA Enhances Immunization with Suboptimal Doses of rHBsAg

Groups of 8 mature adult CF-1 mice were immunized with either 0.5  $\mu$ g or 0.1  $\mu$ g rHBsAg in 25  $\mu$ l alum (273  $\mu$ g/ml), subcutaneously in a single hind footpad. Four mice in each group were pre-treated with either 10  $\mu$ g DHEA in ethanol or the ethanol vehicle alone, topically applied to the same site as the immunization. Serum samples from individual mice were collected and analyzed for specific antibody, as described above. The scattergram shows the responses in ng/ml of each mouse to the vaccine at 28 days post-immunization. FIG. 12 shows that the antibody response to rHBsAg was enhanced for the suboptimal doses of 0.5  $\mu$ g rHBsAg (FIG. 12A) and of 0.1  $\mu$ g rHBsAg (FIG. 12B).

## EXAMPLE 12

## Topical Administration of DHEA Enhances Serum Antibody Production in Elderly Mice Upon Vaccination

Examples 2 and 3 show that DHEA-S administration restored the ability of aged mice to mount a humoral immune response against ovalbumin. This example demonstrates that topical administration of DHEA prior to vaccination against several different antigens enhances serum antibody production in elderly mice.

Aged mice [C3H/HeN], approximately 22–27 months of age, were given a topical application of 10 µg DHEA three hours prior to vaccination with Diphtheria toxoid (Dr, 1.0 µg), Tetanus toxoid (Tt, 1.0 µg), or a Hemophilus Influenza Type b conjugate vaccine coupled to Dt (500 ng of Hib polysaccharide chemically coupled to 1.25 µg Dt) in standard alum adjuvant. Untreated aged mice and untreated mature adult mice [C3H/HeN], 17–24 weeks of age, were administered the ethanol vehicle without DHEA. Serum samples were collected from individual mice at multiple times during the primary response. Individual serum samples were evaluated by quantitative ELISA as described above, using purified Dt (diluted in 0.05M Tris-HCl (pH 9.6) at a concentration of 2.0 µg/ml), purified Tt (diluted in 0.05M Tris-HCl (pH 9.6) at a concentration of 2 µg/ml), or Hib-meningococcal protein conjugate (diluted in 0.05M Tris-HCl (pH 9.6) at a concentration of 200 ng/ml polysaccharide). The mean primary antibody response at Day 28 is shown in Table 1. The results show that the serum antibody responses to these antigens were enhanced in aged mice with topical administration of DHEA prior to vaccination. Similar results are obtained when DHEA is incorporated in the vaccine.

TABLE 1

Immunogen	Serum Antibody Production		
	Primary Antibody Response (µg/ml)		
	Mature Adult	Aged	DHEA-treated Aged
Diphtheria Toxoid (1.0 µg)	1.65	0.43	5.75
Tetanus Toxoid (1.0 µg)	6.8	2.3	4.6
Hemophilus Influenza Type b Conjugate Vaccine:			
Hib Polysaccharide (500 ng)	13.3	6.4	16.2
Diphtheria Toxoid (1.25 µg)	5.8	3.5	7.8

## EXAMPLE 13

## Administration of DHEA-S in Vaccine Enhances Serum Antibody Production in Elderly Mice

Examples 2 and 3 showed that DHEA-S administration restored the ability of aged mice to mount a humoral immune response against ovalbumin. Example 12 demonstrated that topical administration of DHEA prior to immunization enhanced the production of serum antibodies in elderly mice. This Example demonstrates that administra-

tion of DHEA-S in the antigen vehicle also enhances serum antibody production in elderly mice.

Aged mice [C3H/HeN], 22–27 months of age, were vaccinated with inactivated Influenza A Beijing strain (0.1 µg) in a standard alum adjuvant (25 µl; 273 µg alum/ml) which also contained 10 µg DHEA-S. Untreated aged mice and untreated mature adult mice [C3H/HeN], 17–24 weeks of age, were administered the vaccine without DHEA-S. Serum samples were collected from individual mice at multiple times during the primary response. Individual serum samples were evaluated by quantitative ELISA as described above, using purified inactivated Influenza A (diluted in 0.05 M Tris-HCl (pH 9.6) at a concentration of 0.1 µg/ml). The mean primary antibody response at Day 28 is shown in Table 2. The results show that the serum antibody response to this antigen was enhanced in aged mice with incorporation of DHEA-S in the vaccine.

TABLE 2

Immunogen	Serum Antibody Production		
	Primary Antibody Response (µg/ml)		
	Mature Adult	Aged	DHEA-treated Aged
Inactivated Influenza A Virus (0.1 µg)	4.7	0.16	4.3

## EXAMPLE 14

Administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> With Vaccination to Diphtheria Toxoid Enhances Antibody Response

Groups of 3–5 age-matched C3H/HeN female mice were given primary immunization subcutaneously in the right footpad with 10 µg Diphtheria toxoid (Dr; Connaught Laboratories) in alum (273 µg/ml). One group of mice were administered 2µg 1,25(OH)<sub>2</sub>D<sub>3</sub> epicutaneously to the right footpad surface on Day 0. A second group of mice was similarly administered 1,25(OH)<sub>2</sub>D<sub>3</sub> on Day 5 after immunization. A third group of mice (control) received an equal volume of the ethanol carrier. After weekly sampling of serum, mice were secondarily immunized subcutaneously through an intrapelvic route with Dt and no additional exposure to 1,25(OH)<sub>2</sub>D<sub>3</sub>. Serum samples were collected and all primary and secondary samples were then assayed individually using a Dt-specific, quantitative ELISA for IgG and IgA, using purified Dt diluted in 0.05 M Tris-HCl (pH 9.6) at a concentration of 2.0 µg/ml. The purified Dt was dispensed into 96-well plates. Following incubation for a minimum of two hours at room temperature, or overnight at 4° C., all plates were blocked with PBS-0.05% Tween 20/1.0% bovine serum albumin (BSA) for an additional two-hour incubation at room temperature. Prior to adding the test samples, the plates were washed free of blocking buffer using three washes of distilled water and one wash with PBS/0.05% Tween 20. Individual samples were first diluted in PBS 0.05% Tween 20/1.0% BSA and 100 µl was then dispensed into appropriate wells of the antigen-coated plates. Included on each plate was an Ig standard: a series of two-fold dilutions of either purified IgG (all subclasses) or IgA (reference standards). The reference Ig were captured by goat anti-murine Ig which is known to bind all murine Ig isotypes. These plates were incubated at room temperature

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overnight, followed by 3x wash in distilled water and one wash in PBS/0.05% Tween 20. The detection antibody (HRP-lined goat anti-mouse Ig specific for IgG and IgA) was diluted in PBS/Tween/10% normal goat serum at a dilution recommended by the manufacturer. After a final incubation and wash series, the ELISA was developed using ABTS-substrate. O.D. readings were recorded at 405 nM using a Vmax 96-well microtest plate spectrophotometer (Molecular Devices, Menlo Park, Calif.). A simple linear regression analysis of the Ig titration generated a reference curve for calculating the amount of specific antibody contained in the test samples. These data are reported as ng/ml  $\pm$ SEM. It was found that mice which received 1,25(OH)<sub>2</sub>D<sub>3</sub> at Day 0 showed a slight enhancement in serum levels of IgG. Mice which received 1,25(OH)<sub>2</sub>D<sub>3</sub> at Day 0 or Day 5 showed a substantial enhancement in serum levels of IgA. Substantial enhancements in serum IgG and IgA were seen when mice were immunized with Hemophilus Influenza type b polysaccharide conjugate vaccine, Hib coupled to D1 (HibCV) and received 1,25(OH)<sub>2</sub>D<sub>3</sub> on Day 5. Similar results were obtained with topical administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> to other antigens, as shown in the following Example.

## EXAMPLE 15

Administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> Enhances Antibody Response to Vaccinations with Various Antigens

Sex- and age-matched mice [CF-1] were immunized subcutaneously with the following antigens in alum (273  $\mu$ g/ml):

Chlamydia trachomatis peptide (5  $\mu$ g)  
Hemophilus Influenza untypeable (1.0  $\mu$ g)  
Hemophilus Influenza Type b conjugate vaccine coupled to D1 (500 ng of Hib polysaccharide chemically coupled to 1.25  $\mu$ g Dr)  
Respiratory syncytial virus peptide (1  $\mu$ g)  
Hepatitis B Surface Antigen (1  $\mu$ g)  
HIV gp 120 (0.5  $\mu$ g)  
Neisseria gonorrhoeae pilin protein (1  $\mu$ g)  
Diphtheria toxoid (1  $\mu$ g)

One group of mice was administered 2  $\mu$ g 1,25(OH)<sub>2</sub>D<sub>3</sub> epicutaneously at the same site on Day 0. Untreated mice were administered the ethanol vehicle without 1,25(OH)<sub>2</sub>D<sub>3</sub>. Serum samples and mucosal samples (vaginal lavage samples (75  $\mu$ l of physiological saline)) were collected from individual mice at multiple times during the primary response. Individual serum and mucosal samples were evaluated by quantitative ELISA as described in Example 14, using the appropriate antigens. The mean primary antibody responses at Day 28 are shown in Table 3 for the serum (systemic or humoral) antibodies and in Table 4 for the mucosal antibodies. The results show that the serum and mucosal antibody responses were enhanced in mice with topical administration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Mucosal antibodies (both IgG and IgA) were also detected in other mucosal secretions, including lacrimal, rectal, oral and lung. Similar results are obtained when all trans-retinoic acid is used in place of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

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TABLE 3

	Secretory Antibody Production			
	Systemic Ig (ng/ml)			
	Vaccine Only		Vaccine With Topical 1,25(OH) <sub>2</sub> D <sub>3</sub>	
	IgG	IgA	IgG	IgA
<i>Chlamydia trachomatis</i> peptide (5 $\mu$ g)	<0.02	43	55	85
Hemophilus Influenza untypeable	3235	69	4712	93.2
Hemophilus Influenza type b-CV:				
Hib polysaccharide (500 ng)	25	12.5	125	76.1
Diphtheria toxoid (1.25 $\mu$ g)	853	11.6	1285	20.3
Respiratory Syncytial virus peptide (1 $\mu$ g)	2746	225	8440	754
Hepatitis B surface antigen (1 $\mu$ g)	254	58	902	149
HIV gp120 (.5 $\mu$ g)	1356	854	2459	1408
<i>Neisseria gonorrhoeae</i> pilin protein (1 $\mu$ g)	844	16.3	1841	29.1
Diphtheria toxoid (1 $\mu$ g)	1233	23	1640	137

TABLE 4

	Secretory Antibody Production			
	Mucosal Ig (pg/ml)			
	Vaccine Only		Vaccine With Topical 1,25(OH) <sub>2</sub> D <sub>3</sub>	
	IgG	IgA	IgG	IgA
<i>Chlamydia trachomatis</i> peptide (5 $\mu$ g)	<20	397	759	2234
Hemophilus Influenza untypeable	719	1384	2081	1865
Hemophilus Influenza type b-CV:				
Hib polysaccharide (500 ng)	180	440	280	1720
Diphtheria toxoid (1.25 $\mu$ g)	310	400	590	1400
Respiratory Syncytial virus peptide (1 $\mu$ g)	<20	<20	1544	1264
Hepatitis B surface antigen (1 $\mu$ g)	<20	<20	450	250
HIV gp120 (.5 $\mu$ g)	35	45	1428	755
<i>Neisseria gonorrhoeae</i> pilin protein (1 $\mu$ g)	6341	1063	10235	5486
Diphtheria toxoid (1 $\mu$ g)	<20	60	1.8	1125

The above example was repeated, using 0.1  $\mu$ g 1,25(OH)<sub>2</sub>D<sub>3</sub> in the vaccine (in a total volume of 25  $\mu$ l with alum (250  $\mu$ g/ml)) instead of topical administration of the 1,25(OH)<sub>2</sub>D<sub>3</sub>. Identical results were obtained as set forth in Tables 3 and 4.

## EXAMPLE 16

Comparative Effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> and All Trans-Retinoic Acid on Immunoalobulin Production

Groups of sex- and age-matched mice [CF-1], 17-24 weeks of age, were immunized with 1.0  $\mu$ g HBSAg in 25  $\mu$ l alum. The mice in each group were immunized with either

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vaccine alone, vaccine with 0.1  $\mu\text{g}$  1,25(OH) $_2$ D $_3$ , or vaccine with 5  $\mu\text{l}$  all trans-retinoic acid. The agents were incorporated directly into the vaccine mixture. Individual serum (systemic) samples and mucosal samples (vaginal lavage samples (75  $\mu\text{l}$  of physiological saline)) were collected at weekly intervals during the primary response. The mean quantities of antibodies (IgG and IgA) detected in the serum and mucosal secretions 28 days after a single immunization are shown in Table 5. The results show that both 1,25(OH) $_2$ D $_3$  and all trans-retinoic acid enhance both the serum and mucosal antibody response.

TABLE 5

Composition of Vaccine	Antibody Production with 1,25(OH) $_2$ D $_3$ or All Trans-Retinoic Acid			
	Systemic Ig (ng/ml)		Mucosal Ig (ng/ml)	
	IgG	IgA	IgG	IgA
Vaccine only	225	160	35	<20
Vaccine w/ 0.1 $\mu\text{g}$ 1,25(OH) $_2$ D $_3$	457	494	352	341
Vaccine w/ 5 $\mu\text{g}$ All Trans-Retinoic Acid	295	531	437	311

## EXAMPLE 17

Administration of DHEA and 1,25(OH) $_2$ D $_3$  in Vaccine Enhances Serum and Mucosal Antibody Response

Groups of five mature adult C3H mice were immunized with 0.1  $\mu\text{g}$  inactivated Influenza-A Beijing strain in 25  $\mu\text{l}$  of alum (273  $\mu\text{g}/\text{ml}$ ) in the hind footpad. The mice in each group were immunized with either vaccine alone, vaccine plus 2  $\mu\text{g}$  DHEA, vaccine plus 0.1  $\mu\text{g}$  1,25(OH) $_2$ D $_3$  or vaccine with both 2  $\mu\text{g}$  DHEA and 0.1  $\mu\text{g}$  1,25(OH) $_2$ D $_3$ . The agents were incorporated directly into the vaccine mixture. Individual serum (systemic) samples and mucosal samples (vaginal lavages (75  $\mu\text{l}$  physiological saline)) were collected at weekly intervals during the primary response and evaluated by quantitative ELISA. FIGS. 13A, 13B, 13C and 13D show the mean quantities of antibody detected in serum and mucosal secretions 28 days after a single immunization, respectively. The results show that coadministration of DHEA and 1,25(OH) $_2$ D $_3$  in the vaccine synergistically enhances both the serum and mucosal antibody response.

## EXAMPLE 18

Administration of DHEA and 1,25(OH) $_2$ D $_3$  in Vaccine Enhances Serum and Mucosal Antibody Response

Groups of five mature adult CF1 mice were immunized with 1.0  $\mu\text{g}$  rHBSAg in 25  $\mu\text{l}$  of alum (273  $\mu\text{g}/\text{ml}$ ) in the hind footpad. The mice in each group were immunized with either vaccine alone, vaccine plus 2  $\mu\text{g}$  DHEA, vaccine plus 0.1  $\mu\text{g}$  of 1,25(OH) $_2$ D $_3$ , or vaccine with both 2  $\mu\text{g}$  DHEA and 0.1  $\mu\text{g}$  1,25(OH) $_2$ D $_3$ . The agents were incorporated directly into the vaccine mixture. Individual serum (systemic) samples and mucosal samples (vaginal lavages (75  $\mu\text{l}$  of physiological saline)) were collected at weekly intervals during the primary response. FIGS. 14A, 14B, 14C and 14D show the mean quantities of antibody detected in serum and mucosal secretions 21 days after a single immunization. The results show that coadministration of DHEA and

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1,25(OH) $_2$ D $_3$  in the vaccine synergistically enhances both the serum and mucosal antibody response.

## EXAMPLE 19

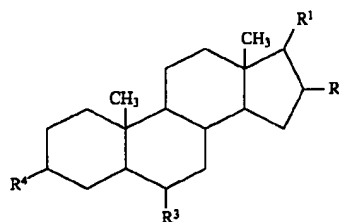
## Administration of DHEA and All Trans-Retinoic Acid in Vaccine Enhances Serum and Mucosal Antibody Response

Groups of five mature adult CF1 mice were immunized with 1.0  $\mu\text{g}$  rHBSAg in 25  $\mu\text{l}$  of alum (273  $\mu\text{g}/\text{ml}$ ) in the hind footpad. The mice in each group were immunized with either vaccine alone, vaccine plus 5.0  $\mu\text{g}$  of all trans-retinoic acid, or vaccine with both 2  $\mu\text{g}$  DHEA and 5.0  $\mu\text{g}$  all trans-retinoic acid. The agents were incorporated directly into the vaccine mixture. Individual serum (systemic) samples and mucosal samples (vaginal lavages (75  $\mu\text{l}$  of physiological saline)) were collected at weekly intervals during the primary response. FIGS. 15A, 15B, 15C and 15D show the mean quantities of antibody detected in serum and mucosal secretions 21 days after a single immunization. The results show that coadministration of DHEA and all trans-retinoic acid in the vaccine synergistically enhances both the serum and mucosal antibody response.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

What is claimed is:

1. A method for enhancing an antigen-specific circulating antibody immune response which comprises administering to an individual a vaccine and an effective amount of an immunomodulator as a vaccine adjuvant, said immunomodulator selected from the group consisting of a lymphoid organ modifying agent and a mixture of all immune response augmenting agent and said lymphoid organ modifying agent, said immune response augmenting agent having the formula



wherein

R $^1$  is =O;

R $^2$  is H or halogen;

R $^3$  is H with a 5-6 double bond;

R $^4$  is OR $^5$ ;

R $^5$  is H, SO $_2$ OM, or PO $_2$ OM;

M is H, Na, K or  $-\text{CH}_2-\text{CH}(\text{O}-\text{CO}-\text{R}^6)-\text{CH}_2-\text{O}-\text{CO}-\text{R}^6$ ; and  
 $\text{O}-\text{CO}-\text{R}^7$

R $^6$  and R $^7$  may be the same or different and may be a straight or branched C $_{1-14}$  alkyl, and said lymphoid organ modifying agent is selected from the group

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consisting of 1,25-dihydroxy Vitamin D<sub>3</sub> and all trans-retinoic acid.

2. The method of claim 1 wherein the immunomodulator is administered up to three hours prior to administration of said vaccine.

3. The method of claim 2 wherein said vaccine comprises an antigen which is capable of eliciting an immune response against viral hepatitis, influenza, diphtheria, tetanus, pertussis, measles, mumps, rubella, polio, pneumococcus, herpes, respiratory syncytial virus, haemophilus influenza type b, varicella-zoster virus or rabies.

4. The method of claim 1 wherein the immunomodulator and vaccine are administered contemporaneously.

5. The method of claim 4 wherein said vaccine comprises an antigen which is capable of eliciting an immune response against viral hepatitis, influenza, diphtheria, tetanus, pertussis, measles, mumps, rubella, polio, pneumococcus, herpes, respiratory syncytial virus, haemophilus influenza type b, varicella-zoster virus or rabies.

6. The method of claim 1 wherein the immunomodulator is administered in a vaccine.

7. The method of claim 6 wherein said vaccine comprises an antigen which is capable of eliciting an immune response against viral hepatitis, influenza, diphtheria, tetanus, pertussis, measles, mumps, rubella, polio, pneumococcus, herpes, respiratory syncytial virus, haemophilus influenza type b, varicella-zoster virus or rabies.

8. The method of claim 1 wherein wherein said immunomodulator is a lymphoid organ modifying agent.

9. The method of claim 8 wherein said lymphoid organ modifying agent is 1,25-dihydroxy Vitamin D<sub>3</sub>.

10. The method of claim 8 wherein said lymphoid organ modifying agent is all trans-retinoic acid.

11. The method of claim 8 wherein the amount of said lymphoid organ modifying agent is 0.1–500 µg.

12. The method of claim 1 wherein wherein said immunomodulator is said mixture of an immune response augmenting agent and a lymphoid organ modifying agent.

13. The method of claim 12, R<sup>1</sup> is =O, R<sup>2</sup> is H, R<sup>3</sup> is H with a 5–6 double bond, and R<sup>4</sup> is OH.

14. The method of claim 13 wherein said lymphoid organ modifying agent is 1,25-dihydroxy Vitamin D<sub>3</sub>.

15. The method of claim 13 wherein said lymphoid organ modifying agent is all trans-retinoic acid.

16. The method of claim 12, R<sup>1</sup> is =OH, R<sup>2</sup> is H, R<sup>3</sup> is H with a 5–6 double bond, and R<sup>4</sup> is OH.

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17. The method of claim 16 wherein said lymphoid organ modifying agent is 1,25-dihydroxy Vitamin D<sub>3</sub>.

18. The method of claim 16 wherein said lymphoid organ modifying agent is all trans-retinoic acid.

19. The method of claim 12, R<sup>1</sup> is =O, R<sup>2</sup> is H, R<sup>3</sup> is H with a 5–6 double bond, and R<sup>4</sup> is OH.

20. The method of claim 19 wherein said lymphoid organ modifying agent is 1,25-dihydroxy Vitamin D<sub>3</sub>.

21. The method of claim 19 wherein said lymphoid organ modifying agent is all trans-retinoic acid.

22. The method of claim 12, R<sup>1</sup> is =O, R<sup>2</sup> is H, R<sup>3</sup> is H with a 5–6 double bond, and R<sup>4</sup> is OH.

23. The method of claim 22 wherein said lymphoid organ modifying agent is 1,25-dihydroxy Vitamin D<sub>3</sub>.

24. The method of claim 22 wherein said lymphoid organ modifying agent is all trans-retinoic acid.

25. The method of claim 12 wherein said lymphoid organ modifying agent is 1,25-dihydroxy Vitamin D<sub>3</sub>.

26. The method of claim 12 wherein said lymphoid organ modifying agent is all trans-retinoic acid.

27. The method of claim 12 wherein the amount of said immune response augmenting agent is 0.1–1,000 µg.

28. The method of claim 27 wherein the amount of said lymphoid organ modifying agent is 0.1–500 µg.

29. The method of claim 12 wherein the amount of said lymphoid organ modifying agent is 0.1–500 µg.

30. The method of claim 1 wherein said immunomodulator is administered epicutaneously.

31. The method of claim 1 wherein said immunomodulator is administered intramuscularly.

32. The method of claim 1 wherein said immunomodulator is administered intradermally.

33. The method of claim 1 wherein said immunomodulator is administered subcutaneously.

34. The method of claim 1 wherein the immunomodulator is administered up to three hours following administration of the vaccine.

35. The method of claim 34 wherein said vaccine comprises an antigen which is capable of eliciting an immune response against viral hepatitis, influenza, diphtheria, tetanus, pertussis, measles, mumps, rubella, polio, pneumococcus, herpes, respiratory syncytial virus, haemophilus influenza type b, varicella-zoster virus or rabies.

36. The method of claim 1 wherein said immunomodulator is administered topically.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 5,562,910

DATED : October 8, 1996

INVENTOR(S) : Raymond A. Daynes et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 16, " $R^2$  is H" should read --  $R^2$  is Br --

In claim 19, " $R^4$  is OH" should read --  $R^4$  is  $OSO_2M$  --

In claim 22, " $R^4$  is OH" should read --  $R^4$  is  $OPO_2M$  --

In claim 16, " $R^1$  is -OH," should read --  $R^1$  is  $=O$ ,--

Signed and Sealed this  
Seventh Day of April, 1998



Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks